In vitro studies to assess the potential of Quercetin as a topical sunscreen; photooxidative properties, photostability and inhibition of UV radiation-mediated skin damage

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by

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Abstract

Protection from the negative effects of solar radiation can be achieved by wearing protective clothing, avoiding exposure to sunlight or by the application of topical sunscreens. In this thesis, a number of studies were designed to determine if quercetin is suitable for use as a topical sunscreen.

The first objective was to determine if quercetin could protect against UV-induced lipid oxidation. Quercetin is twice as effective at preventing UVB-induced oxidation as preventing UVA-induced oxidation.The difference between UVA- and UVB- induced oxidation is believed to be due to the presence of an excited state form of quercetin in the UVA system.

The second objective was to determine the UV photostability of quercetin in solution. Three photoproducts of quercetin form regardless of whether UVA or UVB radiation is used. These photoproducts are 2,4,6-trihydroxybenzaldehyde, quercetin depside and hydroxytyrosol. . The slow rate of formation, less than 20% loss of starting material over 11 hours, and non-toxic nature of the photoproducts indicate that photostability of quercetin is not an obstacle to its use as a sunscreen.

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The fourth objective was to determine if quercetin can prevent UV-induced damage in a biological system. Quercetin was found to significantly reduce secretion of matrix metalloprotease 1 (MMP-1) upon UVA or UVB exposure, but had no effect on secretion

of tumor necrosis factor α (TNF-α) in HaCaT cells. , Topical application of quercetin to UVA or UVB exposed EpiDerm skin mimics significantly reduced both MMP-1 and TNF-α secretion.

These results indicate that quercetin is effective in decreasing or eliminating several harmful effects of UVA and UVB radiation in the skin without major loss of starting material and without formation of toxic photoproducts. As such, quercetin appears to be a good candidate for inclusion into topical sunscreen formulations.

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Dedication

For my wife, Leah. It’s finally done.

List of Figures

List of Abbreviations

4-HNE = 4-Hydroxy Nonenal

8-oxoG = 8-Hydroxy Guanine

AGE = Advanced Glycation End-products

BCC = Basal Cell Carcinoma

C = Cytosine

CPD = Cyclo-pyrimidine Dimer

DMEM = Dulbecco's Modified Eagle Medium

EGF = Epithelial Growth Factor

ELISA = Enzyme Linked Immunosorbent Assay

EMR = Electromagnetic Radiation

G = Guanine

GSH = Reduced Glutathione

IL = Interleukin

LD_{50} = Lethal Dose 50%

MMP-1 = Matrix Metalloprotease 1

NMSC = Non-melanomic Skin Carcinoma

NSAID = Non-steroidal Anti-inflammatory

PABA = Para-amino Benzoic Acid

ROS = Reactive Oxygen Species

SCC = Squamous Cell Carcinoma

SOD = Superoxide Dismutase

SPF = Sun Protection Factor

SSR = Simulated Solar Radiation

T = Thymine

T<>C = Thymine-Cytosine Cyclo-pyrimidine dimer

T<>T = Thymine-Thymine Cyclo-pyrimidine dimer

T(6-4)C = Thymine-Cytosine 6-4 dimer

T(6-4)T = Thymine-Thymine 6-4 dimer

TNF-α = Tumor Necrosis Factor α

UCA = Urocanic Acid

UVA = Ultraviolet A

UVB = Ultraviolet B

UVC = Ultraviolet C

UVR = Ultraviolet Radiation

1. Abstract

Protection from the negative effects of solar radiation can be achieved by wearing protective clothing, avoiding exposure to sunlight or by the application of topical sunscreens. In this thesis, a number of studies were designed to determine if quercetin is suitable for use as a topical sunscreen.

The first objective was to determine if quercetin could protect against UV-induced lipid oxidation. Quercetin is twice as effective at preventing UVB-induced oxidation as preventing UVA-induced oxidation.The difference between UVA- and UVB- induced oxidation is believed to be due to the presence of an excited state form of quercetin in the UVA system.

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These results indicate that quercetin is effective in decreasing or eliminating several harmful effects of UVA and UVB radiation in the skin without major loss of starting material and without formation of toxic photoproducts. As such, quercetin appears to be a good candidate for inclusion into topical sunscreen formulations.

2. Literature Review

2.1 Introduction

Due to an aging population and a depletion of atmospheric ozone, the average North American's total lifetime exposure to sunlight, both in the visible and ultraviolet spectrum, is increasing yearly.

Ultraviolet radiation (UVR) produced by the sun and reaching the earth has the ability to interact with biological systems in a number of ways (Section 2.2), some of which are deleterious to the organism in question. These negative effects on biological systems include initiation of both melanomic (Section 2.3.1.1) and non-melanomic (2.3.1.2) skin carcinomas, resulting from effects of UVR in the 280-315 nm (Section 2.3.2) and 315-400 nm (Section 2.3.3) ranges. UVR can also cause production of reactive oxygen species (ROS) in skin (Section 2.3.4.1) leading to a variety of effects. The most notable of these ROS-dependant UVR effects are decreases in the skin's anti-oxidant defences (Section 2.3.4.2), lipid peroxidation which can lead to necrosis (Section 2.3.4.3), induction of a variety of enzyme cascades with various deleterious outcomes (Section 2.3.4.4), and photoaging effects (Section 2.3.4.5). Finally, UVR has been shown to cause photoimmunosuppression in the skin (Section 2.3.5).

Due to the potentially harmful effects of sunlight, especially solar UVR, protection from sunlight has become a significant health concern. The simplest way to prevent UVR damage is to simply avoid sunlight by remaining indoors during peak daylight hours or wearing protective clothing. However, for various reasons, these steps may not always be a desirable option, which has led to the development of a variety of topical skin protectants commonly referred to as sunscreens. A large number of sunscreens are currently commercially available, but these have a number of associated problems such

as a lack of wide spectrum protection and issues with photostability (Section 2.4.1). Due to the shortcomings of the currently available commercial sunscreens and the public's interest in natural products, a number of plant extracts have been and are being investigated for their potential use as sunscreens (Section 2.4.2). However, to date none of these compounds have been successfully brought to market, making this an active area of research.

One natural product that has potential for use as a sunscreen ingredient is the flavanol quercetin (Section 2.5.1.1). Quercetin is known to act as an efficient anti-oxidant, though the efficiency and capacity are not well characterized, and it is susceptible to oxidation, resulting in a number of products (Section 2.5.1.2). Although the chemical oxidation of quercetin is well studied, the photochemistry, beyond the knowledge of strong UVR absorption, has been only minimally studied (Section 2.5.1.3).

Quercetin is a naturally occurring component of many plant species and as a result is found in most diets (Section 2.5.2.1). Due to its common occurrence in edible plants, the absorption (Section 2.5.2.2) and metabolism (Section 2.5.2.3) of quercetin have been well documented. Quercetin has been found to have a number of beneficial effects in animals when ingested including anti-oxidant effects (Section 2.5.3.1) and effects on various enzyme systems (Section 2.5.3.2). In addition, quercetin has been found to be upregulated in some plants in response to UVR exposure, indicating a role in photoprotection. This photoprotective ability has also been demonstrated in animals fed high levels of quercetin (Section 2.5.3.3).

Although the need for effective sunscreens and the demand for natural products to fulfil this need are well known, the natural polyphenol quercetin which has been shown

to have photoprotective properties has mainly been studied as an ingested or injected photoprotectant, with limited information available on its properties as a topical photoprotectant. We propose that quercetin will be an effective topical sunscreen due protection by UVR screening, free radical scavenging and enzyme modulation.

In the studies presented here, quercetin's ability to prevent ROS induced lipid peroxidation, induction of a MMP-1, a protein associated with photoaging, and TNF-α, a protein induced by thymine dimer formation and associated with immunosuppression will be assessed. Quercetin's photostability will also be assessed and the identity of any decomposition products will be determined. In addition the ability of quercetin to prevent drug-associated photosensitization will be investigated using the anti-inflammatory drug ketoprofen as a model system.

2.2. Ultraviolet Radiation and Photochemistry

2.2.1 - Electromagnetic Radiation

The spectrum of electromagnetic radiation (EMR) ranges from very low energy radiowaves to very high energy γ-waves and cosmic rays [1]. Visible light and ultraviolet radiation comprise a small part of the broad spectrum of electromagnetic radiation (EMR). An overview of the electromagnetic spectrum is shown in Figure 2.1.

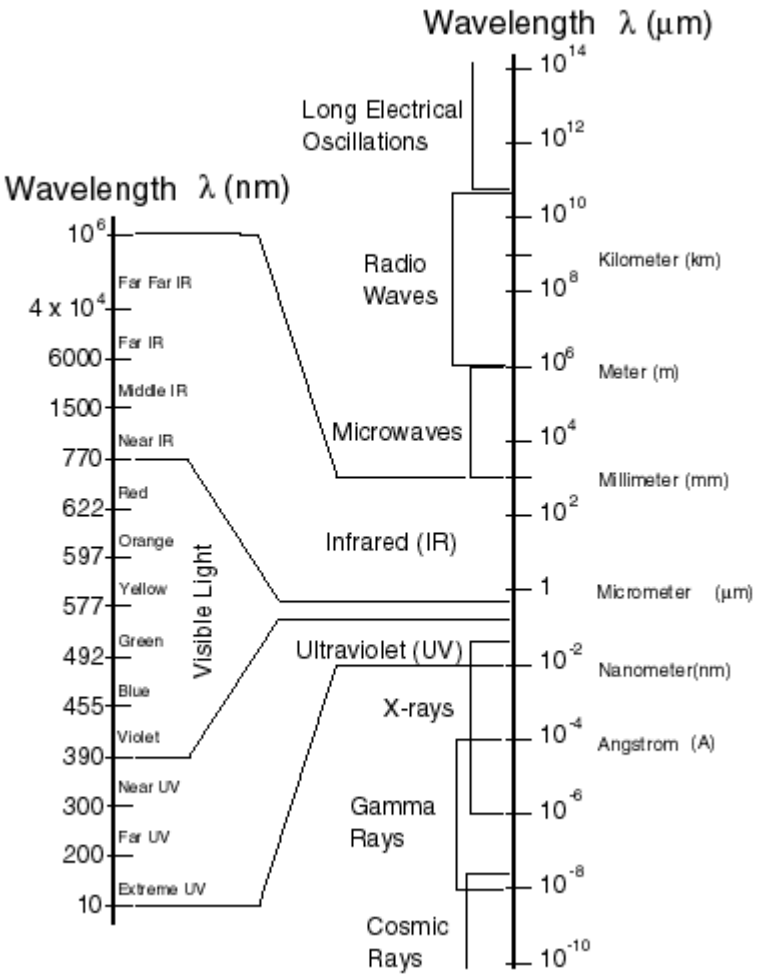


Figure 2.1 - The Electromagnetic Spectrum [2]

The energy of the electromagnetic spectrum posseses the properties of both waves and of particles having no mass, but having momentum [1]. For the purposes of photochemical processes, it is best to consider EMR as a wave of particles as this best explains the interaction of light with matter [3].

EMR is measured using a variety of units such as hertz, wavenumber and wavelength. The units used generally vary with the energy of the EMR being considered, with such units as are convenient being used. For the visible and ultraviolet portions of the electromagnetic spectrum wavelength (λ), defined as the distance between two

consecutive peaks of the EMR, is the most common unit and will be used throughout this thesis.

2.2.2 - Ultraviolet Radiation

Ultraviolet radiation (UVR) is defined as EMR in the range of 100 nm to 400 nm [4] This range is commonly subdivided into three sections: the UVC ranging from 100 nm to 280 nm, the UVB ranging from 280 nm to 315 nm and the UVA ranging from 315 to 400 nm [4]. As the wavelength increases, the energy of the electromagnetic radiation decreases so the energy of UVR is UVC > UVB > UVA.

Of the UVR that reaches the atmosphere of Earth, 100% of the UVC and 90% to 99% of the UVB is absorbed by ozone in the upper atmosphere [5]. As a result the UVR that reaches the surface and is of biological significance is between 1% and 10% UVB, depending on latitude and elevation, with the remainder being in the UVA range [6]. In the United States, the typical total UVR dosage per year ranges from 540000J/m^2 in Bozeman, MT to 970000 J/m^2 in Riverside, CA [5].

2.2.3 - Interaction of Electromagnetic Radiation with Molecules

2.2.3.1 – Chromophores

In order for light to interact with matter, including biological systems, the radiation must interact with a molecule that is capable of absorbing light in the UV range [1]. The portion of a molecule which absorbs EMR is referred to as a chromophore [1] and is responsible for the interaction of matter and light. In the case of UVR chromophores, these consist of π electrons in unsaturated systems, or non-bonding pair electrons such as found on nitrogen or oxygen [1].

The efficiency with which a molecule or portion thereof absorbs EMR is quantified by the molar extinction coefficient or molar absorptivity constant of the absorbing species [3]. The molar extinction coefficient (ε) has units of $L \cdot cm^{-1}mole^{-1}$, and is determined at each wavelength of EMR. Typical ranges of ε for biological molecules in the UV range can vary between 10 $L \cdot cm^{-1}mole^{-1}$ and 30000 $L \cdot cm^{-1}mole^{-1}$, with those molecules having ε values in the 20000 $L \cdot cm^{-1}mole^{-1}$ to 30000 $Lcm^{-1}mole^{-1}$ generally considered as strong chromophores.

2.2.3.2 - Excited Species

Absorption of EMR by molecules results in a change in energy state of the absorbing species to a higher energy level. In the case of radiation in the UV range, the absorbed energy is sufficient for a ground state electron of the absorbing molecule to be raised to a higher energy state, considered an excited state [1]. The electron raised to the higher energy state maintains its quantum spin number (½ or -½), resulting in the excited molecule having paired electrons and initially being in what is termed an excited singlet state [1,3].

The excited species of molecule is generally unstable and will quickly lose energy in order to return to the ground state. This loss of energy, or deactivation, can occur by a number of pathways [1]. The most common pathway of deactivation is internal conversion coupled with vibrational relaxations which involves interaction with other molecules in the sample, resulting in the loss of energy as heat or through transfer of the energy to another molecule. The transfer of energy to another molecule is termed sensitization. A portion of the excitation energy may also be lost due to a process called intersystem crossing, which occurs when the excited electron undergoes a spontaneous

spin change, losing energy in the process. This process results in the formation of a triplet state, which although still an excited state is at lower energy then the excited singlet state. The additional energy is then lost through one of the other deactivation mechanisms. The third possible pathway of energy loss is the emission of a photon, either from the first singlet or first triplet state resulting in fluorescence or phosphorescence, respectively. The final path by which excitation energy may be lost is dissociation in which the excitation energy is high enough to overcome the bond energy in one or more of the bonds of the molecule, resulting in bond cleavage. It is this mechanism of deactivation that may also give rise to photochemical reactions [7]. The pathways by which an excited state electron may lose energy are summarized in a Jablonski diagram in Figure 2.2

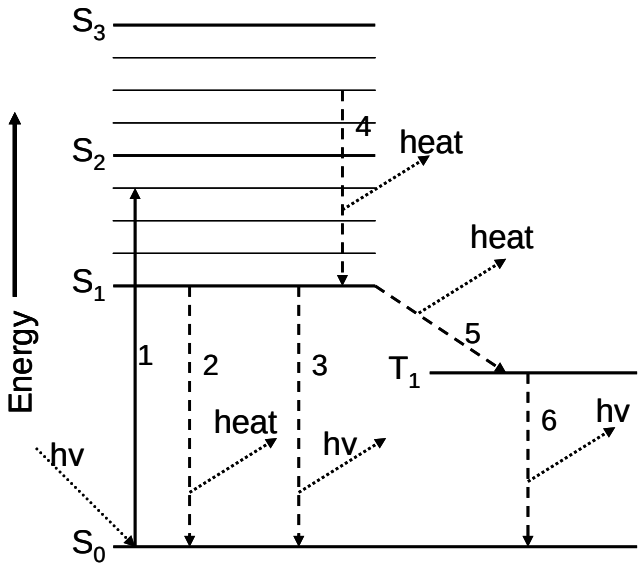


Figure 2.2 - Sample Jablonski diagram showing possible mechanisms of relaxation for an excited state electron. 1 - absorption of a photon, 2 - internal conversion and vibrational relaxation, 3 - fluorescence, 4 - sensitization, 5 - inter-system crossing, 6 - phosphorescence

2.2.4 - Summary

Each day the sun produces large amounts of electromagnetic radiation across the entire spectrum. Only a small portion of this radiation reaches the surface of the earth, primarily radiation in the ultraviolet, visible and infrared ranges. Although this is only a small portion of the sun's total output, it is the ultimate source of the majority of energy consumed on Earth.

In addition to providing the major portion of the Earth's energy, electromagnetic radiation also interacts directly with biological systems through absorption of light. Some of the consequences of this interaction between EMR and biological systems will be discussed in the next section.

2.3. Biological Effects of Ultraviolet Radiation

2.3.1 Skin Cancer

Human skin cancers are a major health concern in North America, Europe and Australia [8-10]. In the United States alone, over a million new cases of skin cancer are diagnosed each year [10,11], accounting for roughly 40% of new cancer cases [9,12,13]. Skin cancer can generally be divided into two broad categories, malignant melanomas which have recently shown a 5% increase each year in North America and Europe [8] and non-melanomic skin carcinomas (NMSC), which are the second most common cancers in the United Kingdom [14,15].

2.3.1.1 Melanoma

Melanomas are, as the name indicates the result of mutations in the skin's melanocytes resulting in uncontrolled proliferation and are highly aggressive [15]. Although melanomas account for only 4% of diagnosed skin cancers, they are more likely to metastasise than NMSC's resulting in a high rate of mortality [10]. In fact,

melanomas account for roughly 8000 deaths per year in the United States [9], and are the most lethal of skin cancers [15]. Melanomas are associated with both the UVA and UVB ranges of solar radiation with UVB causing direct DNA damage and UVA acting through the melanin [8,14,15]. Melanomas are most commonly the result of a single, or very few, intense exposure events resulting in severe sunburn (edema), often occurring early in life [15], and generally present clinically as either a newly formed mole with uneven margins or as changes, such as growth, in a pre-existing mole [16].

2.3.1.2 Non-melanomic Skin Carcinomas (NMSC)

NMSCs account for roughly 96% of the diagnosed cases of skin cancer [15]. These skin cancers result from damage to the keratinocytes, the major component cells of human skin [15]. NMSCs can be further sub-categorized into squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) [12].

2.3.1.2.1 Basal Cell Carcinoma (BCC)

BCCs are the single most common type of skin cancer [10] accounting for roughly 80% of the diagnosed skin cancers in North America [12,15] and Australia [10]. BCC is associated with lifelong exposure to UVB radiation [12], although intermittent exposure during childhood may play a more significant role than exposure later in life [15]. BCC does not have a characteristic precursor lesion [15] and is most commonly found on the head and face as well as other commonly sun-exposed areas of the body [10]. Although several forms of BCC exist, the most common is noduloulcerative BCC which presents clinically as a pearl coloured nodule with a central pit and dilated blood vessels around the edges [10]. BCCs are generally considered to be the least dangerous form of skin

cancer as they do not metastasise [9], but are still a serious problem as they can be locally destructive, and if they occur in exposed areas, are quite disfiguring [10].

2.3.1.2.2 Squamous Cell Carcinoma (SCC)

SSC are the rarer form of NMSCs accounting for 16% to 20% of diagnosed skin cancer [10,12]. Like BCC, SSC is caused by DNA damage to the keratinocyte [15], but unlike BCC is characterized by mutations in the p53 gene [12]. Also unlike BCC, SCC has a characteristic precursor lesion, as most BCC's form from non-cancerous actinic keratoses lesions [15], although SCCs can form *de novo* [10]. SCC is strongly associated with lifelong exposure to UVB which results in the characteristic mutation in exposed tissues [15]. However, a smaller number of SCC's are associated with exposure to the lower energy UVA wavelengths [17,18]. These UVA induced SCC's differ from the UVB induced lesions in that they do not involve mutations to the p53 gene [19] and in that they occur in the dermis rather than the epidermis due to the greater penetrating power of UVA radiation [17]. SCC may present clinically as a thickened scaling patch of skin, a persistent skin ulcer, or a cyst-like nodule [10]. Although not as dangerous as melanomas, SCC's are more invasive and destructive than BCC's and metastasise roughly 10% of the time [9].

2.3.2 UVB Induced Skin Cancer

UVB radiation (280nm-315nm) is the primary wavelength range of light associated with the induction of skin cancer [20-23]. The UVB wavelengths are directly absorbed by the nucleic acids that make up DNA, mainly by the pyrimidine bases thymine and cytosine (and uracil in RNA) [22]

Although the first experiments indicating that UVB could damage DNA used the photosensitizer acetophenone [24], it had since been well established that UVB radiation can cause direct damage to DNA, with the primary lesions being cyclo-pyrimidine dimers (CPDs), especially thymine-thymine CPDs (T<>T) [25-28]. Other lesions which occur due to direct UVB absorption by DNA include thymine-cytosine CPDs (T<>C), thymine-thymine 6-4 photoproducts (T(6-4)T) and thymine-cytosine 6-4 photoproducts (T(6-4)C), along with the Dewer isomers of the 6-4 photoproducts [23,27,29] as shown in Figure 2.3. The relative frequency of direct UVB lesions in fibroblasts was found to be T<>T > T(6-4)C > T<>C > T(6-4)T. [27]

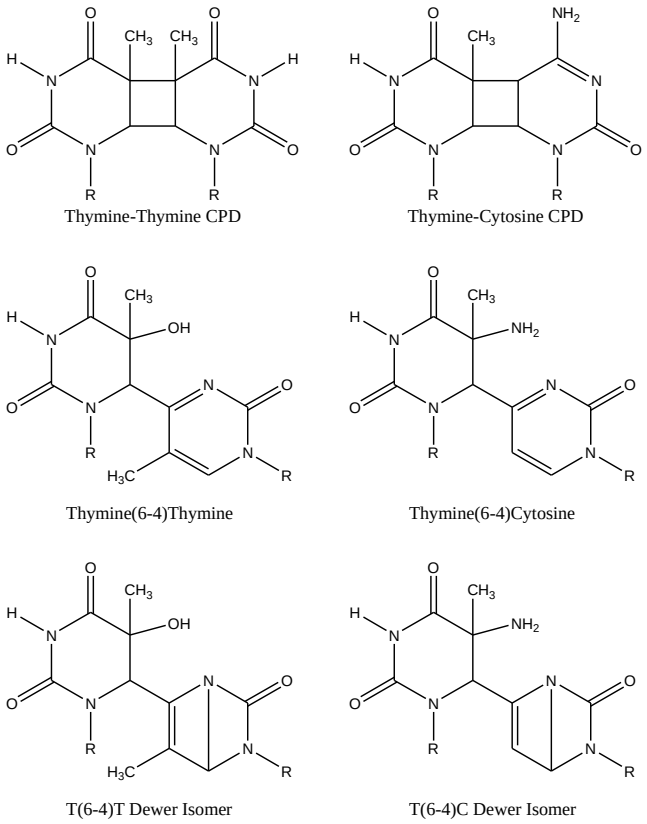


Figure 2.3 - UVB Photoproducts

CPDs form as a result of [2+2] cycloaddition between the C5-C6 double bonds of adjacent pyrimidines [22]. The cis-syn isomer forms more frequently than the trans-syn

isomer, while formation of the cis-anti and trans-anti isomers are sterically impossible in whole DNA [22]. Computational studies have found that the prevalence of the T<>T lesion is due to the fact that this lesion has the lowest excited state energy barrier compared to the other possible lesions, resulting in its favoured formation [21].

In many species, including bacteria, plants and some animals, UVB induced damage is repaired by the UVA activated DNA repair enzyme photolyase [26,30]. However, this enzyme is lacking in humans, necessitating other repair mechanisms [30].

In humans and other mammals, repair of UVR induced CPDs as well as DNA adducts and cross-links are repaired primarily by the nucleotide excision repair (NER) system [11,31]. The proteins responsible for the NER system are the products of at least twenty genes which together carry out five steps required for DNA repair [11,32]. The first step in NER is recognition of DNA damage due to distortions of the DNA double helix. Once the damaged site is recognized, the two DNA strands are separated by two DNA helicases [11]. A 24 to 30 nucleotide long section of the damaged strand containing the damaged bases is then excised. This excision occurs first at the 3' end of the damaged section followed by the 5' end and has an efficiency of 30-40% in 5 to 7 hours [32]. Once excised the damaged section of DNA is removed and the gap is filled by a DNA sythase using the non-damaged strand as a template. The excision strands are then closed by a DNA ligase, and the repair is complete [11]. This repair system is highly conserved in animals, but interindividual variation in efficiencies of up to 20x exist [33].

2.3.3 UVA Induced Skin Cancer

2.3.3.1 Oxidative Lesions

Although it is mainly UVB radiation that is associated with induction of skin cancer, it has been shown that UVA can play a significant role in carcinogenesis as well [8,19,34-36]. Although UVA has a lower energy than the UVB range of solar radiation, UVA accounts for roughly 90% of the UV radiation that reaches the earth's surface, resulting in similar impact of the two ranges of UVR on skin [5,10].

Although the thymine (and to a lesser extent cytosine) residues in DNA provide a good UVB chromophore, and can directly absorb UVB energy [37], the absorption by thymine and other components of DNA in the UVA range is quite weak [37]. As a result, most of the UVA effects on DNA are mediated through ROS, via either type I or type II photoreactions, with oxidized purines outnumbering CPD's by 3 to 1 following UVA exposure [38]

The skin contains a number of compounds capable of absorbing UVA radiation and transferring the energy to molecular oxygen in order to form ROS [39]. Wondrak, et. al. have shown that protein bound advanced glycation end-products (AGE's) can be excited by UVA to cause O_2 dependant and independent DNA cleavage [40]. The same group also showed that unmodified collagen and elastin in the skin as well B6 vitamirs can act as UVA sensitizers to ROS mediated DNA damage [41,42]. Pouget, et al. have shown that the major ROS associated with UVA exposure is $^1O_2$ rather than H_2O_2 [22,43]

As stated earlier, the main DNA lesions caused by UVA exposure are formed via ROS, with the most common lesion being 8-hydroxyguanine (8-oxoG) [22,28,44,45] The result of this alteration to the guanine base is most commonly a deletion mutation in

the complementary strand directly across from the oxidized guanine [46], although G to T transversions may also occur [19].

2.3.3.2 UVA Photoleisons

In addition to oxidative lesions, UVA radiation is also associated with thymine dimer formation [17,36,38,47]. UVA is a much weaker inducer of thymine dimers than UVB, about three orders of magnitude less efficient [35,38], the abundance of UVA results in number of CPD's resulting from the UVA portion of sunlight [17].

Although UVA is much lower in energy than UVB, the greater abundance of UVA and its ability to induce oxidative damage means that UVA poses a significant skin cancer risk at environmental levels [8,17,36]. An interesting aspect of this UVA damaged DNA is the stratification of damage. Due to the greater penetrating power of UVA compared to UVB radiation, DNA damage caused by UVB tends to be found in the upper layers of the skin while UVA damage in occurs basally located cells [17].

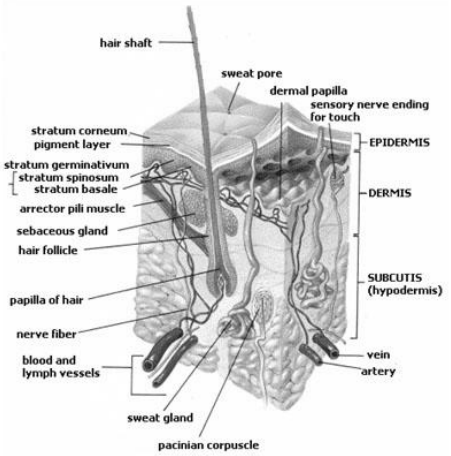


Figure 2.4 - Cross-sectional diagram of human skin showing the three main layers of the skin and the component cells [48]

2.3.4 Oxidative Stress

2.3.4.1 Reactive Oxygen Species Generation

In addition to damage to DNA by direct absorption, UVR may cause other deleterious effects in the skin. The most significant of these damages is the generation of reactive oxygen species in the skin by both UVA [49] and UVB [50] radiation. UVR has been shown to generate a number of different ROS in the skin including the primary species singlet oxygen ($^1O_2$) [49,51-54] and superoxide radical ($O_2^{\cdot -}$) [55-57] and the secondary species hydrogen peroxide (H_2O_2) [41,49,56,58] and hydroxyl radical ($^{\cdot}OH$) [50,56].

The primary ROS are produced through either a type I photoreaction wherein energy is transferred from an excited state chromophore to a target molecule which then reacts with molecular oxygen ($^3O_2$) to generate the ROS or a type II photoreaction where the excited state chromophore transfers energy directly to molecular oxygen to generate the ROS. Predictably, the type II photoreaction is more efficient [59]. Singlet oxygen and superoxide are subsequently converted to H_2O_2 either by superoxide dismutase (SOD) [60] or spontaneously at neutral pH's [61] in the case of $O_2^{\cdot -}$ or anti-oxidants such as ascorbate in the case of $^1O_2$ [62]. H_2O_2 is less reactive than either $^1O_2$ or $O_2^{\cdot -}$, and freely diffuses across membranes, potentially reaching all parts of the cell [63]. H_2O_2 can then be reduced to the highly reactive hydroxyl radical by Fe(II) or Cu(II) present in the cell via a Fenton reaction [61].

In order to generate ROS, UVR must first be absorbed by a molecule in the skin, referred to as a chromophore. A variety of different molecules have been identified as serving as UV chromophores in human skin. One of the most important UVR chromophores in the skin is DNA [37]. However, UV absorption by DNA is generally

associated with DNA lesions rather than the generation of ROS as discussed in sections 2.3.2 and 2.3.3.1.

Another well studied UVR chromophore in skin is urocanic acid (UCA) which is found in high levels in the stratum corneum, the upper most layer of the epidermis. Urocanic acid (Figure 2.5) is produced by the deamination of histidine by histidine ammonia lyase [14]. Urocanic acid exists normally in the skin as the *trans* isomer, but exposure of *trans*-UCA to UVR results in a photoisomerization to *cis*-UCA [14,64]. In addition to the photoisomerization, the absorption of UVR, specifically UVA, by UCA results in the formation of $^1O_2$ [64]. The UCA then reacts with the $^1O_2$ formed to form oxidation products which include species that also absorb UVR increasing the rate of $^1O_2$ and peroxide formation [64].

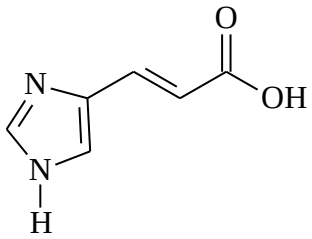


Figure 2.5 - Urocanic acid

Other endogenous small molecules in the skin may also act as photosensitizers. These include bilirubin [65], B vitamers [42] and tryptophan [66] which are all water soluble and contain UVR absorbing π electrons as well as ionizable oxygens which leads to the generation of H_2O_2 when exposed to UVR in the presence of oxygen. Also implicated as photosensitizers are pterins which produce $^1O_2$ in response to UVA [67] and flavins such as riboflavin which produces both $^1O_2$ and $O_2^{\cdot -}$ when exposed to UVR [68]. Finally a variety of porphyrins may also act as photosensitizers [69].

In addition to small molecules and DNA, proteins - both modified and unmodified - can act as UVR chromophores for the generation of ROS. Wondrak, et. al. have shown that simulated solar radiation (SSR) and UVA alone can be absorbed by extracellular matrix (ECM) proteins in the skin such as collagen and elastin, resulting in the production of H_2O_2 which can then enter the cell across the plasma membrane [41]. The photoreductive formation of H_2O_2 by UVA and SSR also results in the oxidation and subsequent fragmentation of the ECM proteins [41]. This same group later showed that the portion of the ECM proteins primarily responsible for the UVR absorption and ROS generation are the hydroxypyridine containing cross-links [42]. It was further demonstrated that the minimum hydroxypyridine derivative sensitizer chromophores are 3-hydroxypyridine for UVB and *N*-alkyl-3-hydroxypyridinium in the case of UVA [42]. In addition to the ECM proteins elastin and collagen, UVB exposure absorption by catalase can also result in the formation of ROS. Although catalase is normally associated with the reduction of H_2O_2 to H_2O, Heck, et. al. have shown that in keratinocyte cultures exposed to UVB, catalase caused the formation of superoxide and subsequently H_2O_2 [70].

Although unmodified ECM proteins can act as UVR sensitizers, modified proteins are generally more effective in this role. AGE's are formed by spontaneous protein damage caused by reactive carbonyl species such as reducing sugars [71]. These protein modifications, many of which have a UVA chromophore consisting of pi electrons, accumulate on long lived proteins such as the skin proteins collagen and elastin during normal skin aging [72]. When exposed to UVA, these protein modifications have been shown to generate a variety of ROS including H_2O_2 [39], $O_2^{\cdot -}$ and $^{\cdot}OH$ [56] in both

keratinocytes [39] and fibroblasts [56]. In addition to generating ROS, AGEs have also been shown to cause cleavage of DNA directly upon absorption of UVR in a Type I photoreaction in cell culture systems [40].

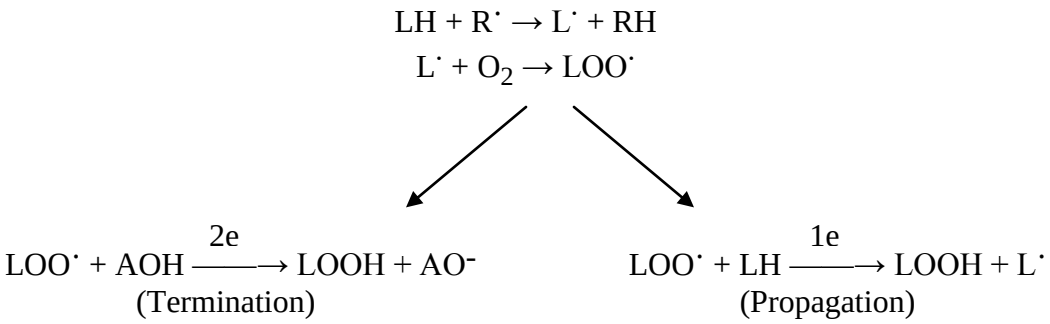
2.3.4.2 Antioxidant status of photoexposed skin

Normal healthy skin serves as the body's first line of defence against a variety of environmental insults and as a result has a number of natural defences, particularly against oxidative stress. These defences include enzymatic defences such as SOD, catalase, glutathione peroxidase and glutathione reductase as well non-enzymatic defences such as ascorbate, glutathione (GSH), α-tocopherol and ubiquinol [63,73,74].

Predictably, the increase in ROS in the skin caused by exposure to UVR can result in a strain on this antioxidant defensive system. Using mice exposed to 25 J/cm^2 of simulated solar light, equivalent to 5 hours of autumn sunlight at 38° N, Yasuko, et. al. showed that glutathione peroxidase and glutathione reductase both showed slight but significant decreases in both the dermis and epidermis following exposure while catalase and SOD showed large decreases in protein levels and activity [75]. In the same study, ubiquinol was found to completely disappear following exposure while α-tocopherol decreased by 30%, ascorbate decreased 53% in the epidermis and 68% in the dermis and GSH decreased, but not significantly, in both dermis and epidermis [75]. The same group later demonstrated similar results using much lower doses of 2 to 12.5 J/cm^2 [73]. These results are supported by the findings of other groups such as Aldini et. al. who showed that UVB exposure decreased GSH levels in human skin cell cultures [76].

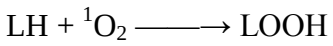
2.3.4.3 Lipid Peroxidation

Following depletion of the skins anti-oxidant defences, continued UVR induced production of ROS leads to a build up of these reactive molecules which can cause a number of types of damage in the skin, such as lipid peroxidation. Lipid peroxidation occurs when a hydrogen atom is abstracted from a methylene carbon in the side chain of a lipid, which is made easier by increasing degrees of unsaturation, by a hydroxyl radical [77]. This abstraction produces a carbon-centred radical which quickly reacts with ground state molecular oxygen to form a peroxyl radical [55,77,78]. This process can then repeat via one-electron reduction of an adjacent lipid resulting in a self propagating chain reaction (scheme 2.1) [78]. Alternatively, interactions with an anti-oxidant species can result in a two-electron reduction, terminating the chain [78,79].



Scheme 2.1

The formation of the lipid peroxide may also be initiated by singlet oxygen, giving rise to the reduced species [78] as shown in scheme 2.2.



Scheme 2.2

It should also be noted that the propagation of the peroxidation seen in scheme 2.1 is not confined to adjacent lipids as the lipid radical species may move within or between membranes [78].

Exposure to UVR has been shown to be linked to lipid peroxidation in a number of systems including epidermis homogenate [50,55] and HeLa cells [80,81]. Electron spin resonance (ESR) studies showing the co-occurrence of UVR induced ROS and lipid peroxidation [50,55] as well as a linear relationship between the dose of UVR, ROS formation and lipid peroxidation [50] indicate a causative role for ROS in UVR induced lipid peroxidation.

The overall effect of these ROS induced lipid peroxides is to alter the structure and function of the membranes in which they occur as they generally have a greater polarity then the parent lipids [78]. One of the alterations seen in oxidized lipid membranes is an increase in rigidity or loss of fluidity. This loss of fluidity can cause a decrease in cell function as membrane bound proteins can no longer move freely or cell death as the membrane becomes leaky [49,55,82].

UVR induced lipid peroxides have also been implicated in a number of secondary effects. One of the products of membrane lipid peroxidation is 4-hydroxynonenal (4-HNE) [83]. 4-HNE has been shown in cultured cells to increase in a dose dependant manner with UVA irradiation, and that the increased levels of 4-HNE, which forms DNA and protein adducts, leads to an increased rate of apoptosis [83]. In addition changes in the fluidity of cellular membranes have been suggested to have an effect on cell surface receptor proteins. Rosette et. al. found that UVB radiation caused multimerization, clustering and internalization of the cell surface receptors for epithelial growth factor

(EGF), tumour necrosis factor (TNF) and IL-1 identical to that seen when the receptors bind to their appropriate ligand, which initiated a series of enzyme cascades within the cells (see section 3.4.4)[80]. These findings are supported by later work which found that UVB radiation can cause the release of IL-6 [84] and activation of the CD95 death receptor [81] in a membrane mediated, DNA independent manner. Although the mechanism is not clear, it has been suggested that changes in membrane fluidity caused by lipid peroxidation are the cause of these clustering effects [80,85].

2.3.4.4 UVR Induced Enzyme Cascades

Although the process leading from the generation of ROS to the activation of various cell surface receptors is not entirely clear [80,85], it is well established that UVA and UVB radiation can initiate a number of enzyme cascades inside the cell [34,51,60,86-88].

UVR exposure of keratinocytes and fibroblasts has been shown to cause the production and release of a number of cytokines. Fibroblasts exposed to UVR have been shown to release IL-1α and IL-1β which then initiate the de novo synthesis of more IL-1α and IL-1β creating a positive feedback loop [89]. IL-1α and IL-1β release has also been demonstrated in stratified epithelium containing both fibroblasts and keratinocytes. This release was correlated to cytotoxicity, and interestingly was not affected by thickness of the skin, likely due to the penetrating power of UVA and UVB radiation. This penetrating power is due to the short wavelength of UVR being such that interference with many skin components does not occur [90].

The UVR induced activation and production of IL-1 has a number of downstream consequences. IL-1 can cause the release and production of IL-6 [89] which has been shown to be increased in UVA exposed fibroblasts [89,91,92], keratinocytes [90], HeLa

cells [84], stratified epithelium [90], and in humans *in vivo* [93], usually in association with IL-1α and IL-1β.

UVR induced activation of the cytokine receptors, either through induction of interleukin release and production [89,90,92,94] or through membrane effect activation [80,85], has also been shown to result in the initiation of a kinase cascade leading to activation of AP-1 [88,95,96]. Activation of cytokine receptors first causes the recruitment of the small GTP-binding proteins Ras [97], and Rac [98]. These proteins in turn activate mitogen activated protein kinase kinase kinase 1 (MAPKKK1 or MEKK1) [99]. In addition to acting thorough membrane effects, intracellular ROS, specifically ·OH may also activate MEKK1 directly [60].

Activated (phosphorylated) MEKK1 in turn phosphorylates mitogen activated protein kinase kinase 4 (MAPKK4 or MEK4) with then phosphorylates MEK7 [60]. MEK7 acts on the c-Jun N-terminal kinase (JNK) protein, activating it through phosphorylation of residues Thr183 and Tyr185, as well as activating the p38 protein by phosphorylation of Thr180 and Tyr182 [86,100]. The activation of JNK and p38 has been shown to occur in fibroblasts [52,53], HaCaT (keratinocyte) cells [101] and photoaged skin [95].

JNK, and to a lesser extent p38, act on the c-Jun protein, phosphorylating it at residues Ser63 and Ser73, which stabilizes c-Jun and increases its transcriptional activity [88,101]. The c-Jun protein is a component of activating protein-1 (AP-1) which is composed of a homodimer of c-Jun or a heterodimer of c-Jun and one of v-Jun, JunB, JunD, v-Fos, c-Fos, FosB, Fra1, Fra2, ATF2, ATF3, B-ATF or bZIP [101]. In addition to the activation of c-Jun, UVR has been shown to induce the expression of the AP-1 components c-Jun, c-Fos, Fra1and Fra2 [96]. The transcriptional activation of AP-1 by JNK mediated

phosphorylation of c-Jun along with the increased expression of AP-1 components results in the up-regulation of AP-1 controlled genes, among which is the *c-Jun* gene, resulting in a further increase in c-Jun levels [88,95,96,101,102].

AP-1 is involved in a number of cellular functions. The c-Jun component of AP-1 appears to be required for growth and development of some cells, especially fibroblasts [102]. However, AP-1 has also been implicated in the initiation of apoptosis, particularly those types of apoptosis requiring *de novo* protein synthesis [102], particularly through the activity of cytochrome-c [103]. The action of UVR induced AP-1 may partially explain the induction of apoptosis associated with UVR [103-105] as well as the findings that UVR induced JNK can prevent apoptosis [106].

AP-1 also acts to activate matrix metalloprotease (MMP) genes, leading to the increase in synthesis and release of MMP-1 and other metalloproteases seen in UVR exposed cells [87,89,90,92,95,107-110]. The UVR induced photoaging effects of AP-1 mediated MMP induction will be discussed in more detail in section 3.4.5. However, it should be noted that UVR can also affect collagen status through regulation of Smad7 activity. AP-1 binds to the promoter of the *Smad*7 gene resulting in an increase in Smad7 transcription, which results in increased translation and activity. This increase in Smad7 results in a decrease in phosphorylation of Smad2 and Smad3 which prevents the synthesis of procollagen-I through modulation of the TGF-β pathway [60,111]. Procollagen –I is converted to collagen-I, so the decrease in procollagen-I, leads to a decrease in collagen-I also contributing to the photoaging process [60]. AP-1 is involved in the regulation in a number of genes besides those described which have been discussed in a number of reviews [102,112].

Exposure of keratinocytes to UVR has also been shown to increase expression of intercellular adhesion molecule 1 (ICAM-1) through the generation of ROS [113-115]. ICAM-1 which is involved in the skin's inflammatory response as well as tumour promotion [113,116], has been shown to be at least partially under the control of activating protein-2 (AP-2) which is upregulated by UVR [114]. AP-2 has been shown to be activated by IL-1α, which as discussed earlier is induced by UVR [115]. In addition, AP-2 may be activated by ceramide which has been shown to be produced from sphingomyelin in a non-enzymatic mechanism involving UVR derived ROS, this reaction presumably occurring at the cell membrane [117].

The preceding UVR induced enzyme cascade description covers the portion of the possible enzyme effects relevant to this project. A more complete description is available in several review articles [34,51,60,86-88]. A graphical presentation of this partial enzyme cascade is shown in figure 2.6.

2.3.4.5 Photoaging

In addition to genetic and oxidative damage, ultraviolet light exposure has also been associated with acceleration of the aging process, referred to as photoaging [15]. Photoaging is characterized by increased wrinkle formation relative to unexposed skin, a loss of skin recoil capacity and an increase in skin fragility [87,91,118]. Histologically, the most prominent feature of photoaged skin is solar elastosis, characterized by degradation of collagen and accumulation of abnormal elastin [118,119].

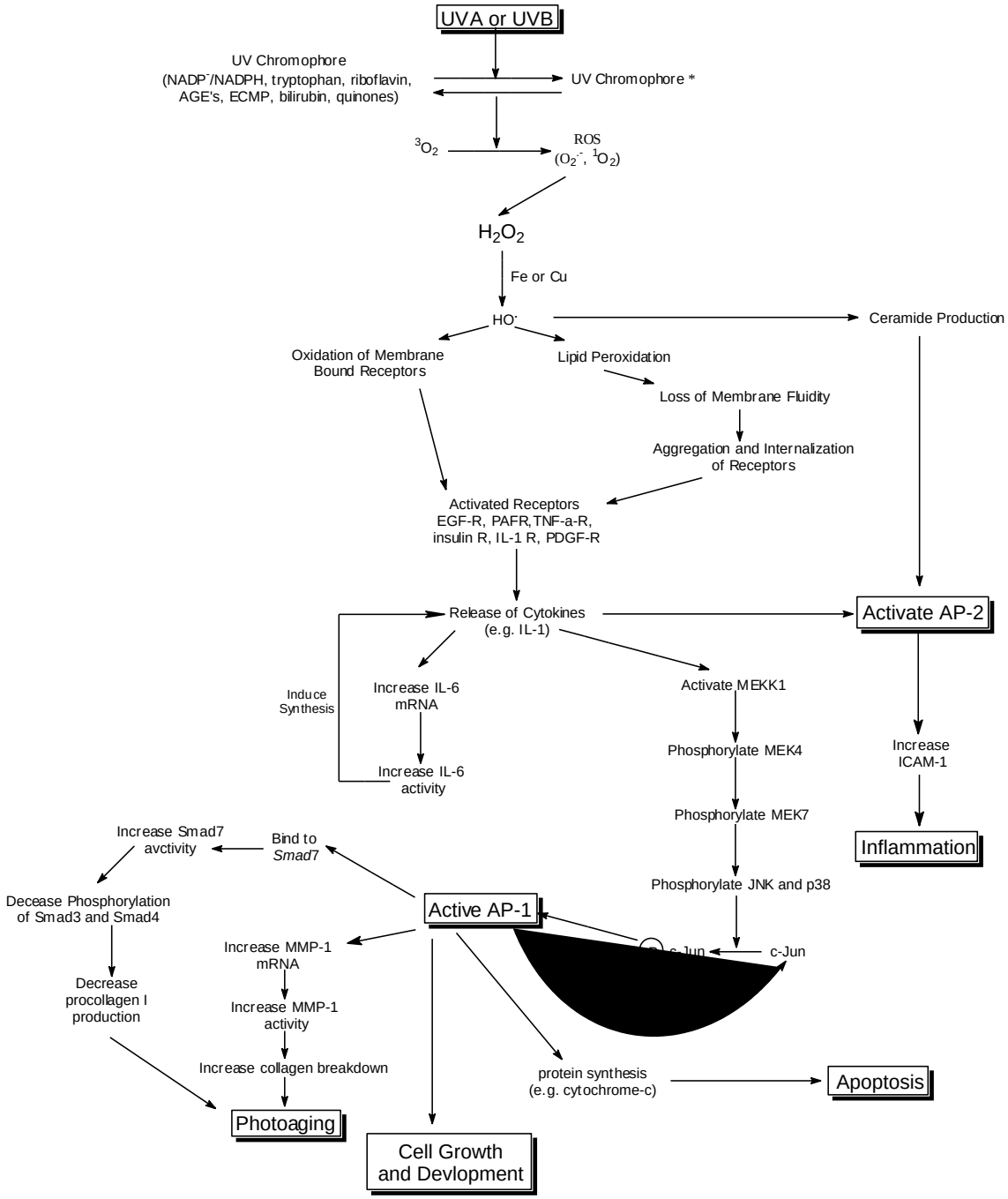


Figure 2.6 - UVR induced enzyme cascades

Collagen is an extracellularly excreted protein that is primarily responsible for providing structural integrity to the skin [108], and loss of this protein is the main cause

of the skin changes seen in photoaging, although changes to other extracellular proteins such as elastin and glycosaminoglycans are also involved [87].

Breakdown of collagen is primarily mediated through the enzyme MMP-1, also known as collagenase, which is responsible for the breakdown of interstitial collagen [108]. It is the modulation of MMP-1 levels by UVA and UVB radiation that is believed to be the main cause of photoaging [87,95,107-110].

The induction of MMP-1 in the skin by ultraviolet radiation has been demonstrated by a number of groups. Using cultured fibroblasts grown from biopsies of healthy donors, Herrmann et. al. showed that UVA radiation at a dose of 180kJ/m^2 could induce MMP-1 mRNA and *de novo* MMP-1 protein synthesis and excretion as well as MMP-3 mRNA and MMP-2 and MMP-3 protein synthesis and excretion up to 48 hours after exposure [120]. This same group had earlier shown that a dose of 10 J/cm^2 could induce MMP-1 mRNA in cultured fibroblasts [110]. These findings are supported by the work of Vielhaver, et. al. which showed that sunscreens which absorb UVA protect against UV induced expression of MMP-1 [92].

Working with human skin in vivo, Fisher et al have shown that UVB doses as low as 10% of the minimal erythema dose (MED) for an individual induce MMP-1 mRNA and protein as well as MMP-9 (gelatinase) mRNA and protein up to 48 hours after exposure in a dose dependant manner [107]. This same group later showed that MMP-1 mRNA was upregulated by UVB in both the dermis and epidermis and that this MMP induction led to degradation of skin collagen [109]. This was supported by Onoue, et. al. who showed that keratinocytes exposed to UVB, but not UVA, showed an increase in MMP-9 excretion [121].

Photoaged skin is distinct from naturally, or chronologically, aged skin in both appearance and in biochemical properties. Superficially, naturally aged skin is smooth, pale and finely wrinkled as compared to coarsely wrinkled photo-aged skin [122]. Biochemically, levels of the matrix-metalloproteases MMP-1, MMP-2 and MMP-9 are all elevated in photoaged skin as compared to naturally aged skin [95,122]. In addition, photoaged skin has a lower expression of the antioxidant enzymes copper-zinc superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD) and catalase (CAT) than naturally aged skin. This results in an increase in oxidative protein damage, specifically the formation of protein carbonyls [119]. Finally, photoaged skin shows changes from naturally aged skin in the collagen balance. In photoaged skin the level of procollagen mRNA and protein expression is increased relative to naturally aged skin, but due to increases in the MMPs, the total amount of functional collagen is decreased in photoaged skin [122].

The balance between synthesis and degradation of collagen is the key factor in determining the extent of photoaging in otherwise healthy, young skin. Comparing sun-protected and sun-exposed skin from healthy young volunteers, Varani et.al. showed that collagen fragmentation was increased in sun-exposed skin [123]. However, in contrast to the comparison between naturally aged and photo-aged skin, photo-damaged skin showed a reduction in pro-collagen synthesis relative to undamaged skin [123]. Varani further found that the inhibition of collagen synthesis was caused, at least in part, by fragmented, or degraded, collagen. This was demonstrated by the fact that fibroblasts removed from an area of sun-damaged skin produced collagen normally when the exposure to collagen fragments ceased [123].

Although the complete molecular pathway leading from exposure to UVR to induction of the matrix-metalloproteases and photoaging is not entirely clear, some research exists concerning the chain of events. It is well known that both UVA and UVB radiation can induce the formation of reactive oxygen species (ROS) in the skin [49,50] and there is good evidence that it is these ROS that initiate the photoaging process. First, the level of H_2O_2, an inducer of MMP-1 [124], has been found to be elevated in photoaged skin relative to naturally aged skin, perhaps accounting for the differences in MMP activities [95]. Secondly, it has been shown that dark reaction produced $^1O_2$, which can be converted to form H_2O_2, induces MMP-1 mRNA in the same dose dependant manner as UVA in cultured fibroblasts [124]. In addition, exposure of fibroblasts to UVA resulting in MMP-1 upregulation has been shown to be enhanced by the addition of D_2O, which increases the lifetime of $^1O_2$ [125], and that addition of sodium azide, a quencher of $^1O_2$, dramatically decreases the expression of MMP-1, implicating $^1O_2$ in the process [126]. Taken together, these facts may show that the formation of reactive oxygen species is the first step in photoaging.

The enzymatic pathway connecting ROS to photoaging has been filled in by a number of groups and appears to involve several interleukins. It has been determined by Wlaschek, et. al. that fibroblasts exposed to UVA show an upregulation in IL-6 mRNA and protein and that inhibition of the translation of IL-6 prevents MMP-1 upregulation, showing that IL-6 is necessary for increases in MMP-1 activity [91]. This same group later showed that following UVA exposure, there is an initial increase in the release of existing IL-1 which triggers the *de novo* synthesis of both IL-6 which leads to MMP-1 induction and IL-1 which perpetuates the UV response [89].

2.3.5 Photoimmunosuppression

In addition to the effects on DNA, lipid membranes and signal cascades, UVB has also been shown to cause immune suppression [15,113]. It is believed that this photoimmunosuppression serves to prevent harmful excessive inflammation of the skin following exposure to UVR [15]. Although this suppression of the immune system occurs first in the skin as expected, it can become systemic [15]. In addition to acute occurrence of immunosuppression, long term exposure to doses of UVB too low to induce acute immunosuppression have been shown to cause chronic, cumulative suppression of the immune system in mice [127].

The initial skin chromophore involved in immunosuppression has been shown to be DNA, specifically the formation of cyclobutane dimers. Yarosh, et. al. demonstrated that in HaCaT cells UVB radiation induces the immunosuppressive cytokine TNF-α, and that this same cytokine was induced when DNA was damaged using *HindIII*. Further, they also showed that increased repair of CPDs by addition of T4 endonuclease V, which is specific for UVR induced DNA damage, resulted in a decrease in immunosuppression

[128]. Similar results have shown that in *in vivo* human experiments, T4 endonuclease V given after SSR exposure prevented immunosuppression [129].

Immunosuppression by UVB occurs via suppression of T-cell mediated immunity [15,130] and is controlled through the induction of immunomodulating cytokines. UVB has been shown to induce the production and release of IL-10 in human keratinocytes [131] and mice [132-134] the blocking of which results in a loss of immunosuppression [131-134]. In addition to IL-10, UVB has also been shown to induce production of IL-4 [132], IL-6 [133] and TNF-α [132] in mice.

The various interleukins induced by UVB radiation appear to have different functions in the immunosuppressive effect. When IL-6 knockout mice are exposed to UVB, the loss of contact hypersensitivity seen in wild type mice as well as the increase in IL-6 and IL-10 levels in the blood were absent [133]. However, when the knockout mice were injected with IL-6, the IL-10 levels increased and immunosuppression was observed, indicating that UVB causes induction of IL-6 which in turn induces IL-10 which results in immunosuppression [133]. This same study indicated that the cells responsible for IL-6 production appeared to be Langerhan's cells [133]. It has also been demonstrated that different UVB induced interleukins modulate different types of immunosuppression. Rivas et. al. showed that injecting UVB exposed mice with anti-IL-10 reduced the effects of UVB on delayed type hypersensitivity but not contact hypersensitivity while anti-TNF-α decreased suppression of contact hypersensitivity but had no effect on delayed type hypersensitivity [132].

UVB radiation is associated with both acute and chronic immunosuppression, but longer wavelength UVA radiation appears to have the opposite effect. Reeve, et. al.

found that mice exposed to pure UVA radiation immediately after or up to 24 hours before exposure to UVB did not show immunosuppression as seen in mice exposed to UVB alone, but did not provide a mechanistic explanation [135]. This same group later showed that in albino hairless mice UVA radiation induced IFN-γ expression peaked at 1 day post-exposure followed by IL-12 expression peaking at 3 days post-exposure while UVB induced IL-10 expression peaked on day 3. Exposure to both UVA and UVB resulted in a lack of IL-10 expression and decreased IL-12 and IFN-γ expression. Since IL-12 is a known agonist of IL-10, the authors concluded that UVA protects from UVB induced immunosuppression by increasing expression of IL-12, likely through IFN-γ expression, which decreases IL-10 expression [134]. Reeve, et.al. then showed that the UVA induced expression of IFN-γ and IL-12 was mediated through the induction of the stress enzyme heme oxygenase-1 [136].

2.3.6 Summary

Exposure to UVR can cause a number of negative biological effects in humans, primarily skin cancer, oxidative stress and immunosuppression. UVR induced skin cancers include the rare but highly malignant melanoma as well as the less malignant but more common non-melanomic skin carcinomas basal cell carcinoma and squamous cell carcinoma.

Skin carcinomas can be induced by both UVB and UVA ranges of solar radiation. UVB radiation can be directly absorbed by DNA which may result in the formation of CPD's, particularly thymine-thymine dimers which can cause replication or transcription errors if not repaired. Although UVA radiation can cause direct formation of thymine dimers in the same manner as UVB, UVA induced skin caner is more often caused by

oxidative DNA lesions. In these cases, UVA radiation absorbed by endogenous or exogenous chromophores in the skin provides the energy for the formation of reactive oxygen species with react with DNA, usually at guanine residues to form lesions. These lesions can then cause replication or transcription errors resulting in uncontrolled cell growth.

In addition to causing oxidative DNA lesions, UVR derived ROS have a number of other negative effects. Due to the harmful effects of ROS, the skin has a number of natural defences to eliminate them, however depletion of these defences by UVR generated ROS can leave the body more vulnerable to ROS from other sources. In addition, UVR generated ROS can initiate lipid peroxidation which may result in loss of membrane function or necrosis. ROS can also initiate a number of enzyme cascades resulting in inflammation, changes in cell growth and development and apoptosis. Finally, enzyme cascades initiated by UVR induced ROS can cause photoaging effects by causing destruction of interstitial collagen.

Finally, UVR has been shown to cause photoimmunosuppression in human skin. This phenomenon is caused by the absorption of UVR (primarily UVB) by DNA resulting in the production of IL-10, IL-4, IL-6 and TNF-α. The induction of these cytokines results in both acute and chronic immunosuppression, both locally and systemically.

It is clear that while necessary for a number of biological functions, solar UVR also has a number of harmful biological effects. In order to reduce these effects and the negative outcomes associated with them it is necessary to reduce the amount of UVR exposure most people receive. One approach to this is the effective use of sunscreens, as described in the next section.

2.4. Sunscreens

2.4.1 Commercial Sunscreens

With the wide variety of deleterious health effects seen with overexposure to ultraviolet radiation (see section 2), a variety of methods to prevent this damage are available. The simplest means of avoiding UVR exposure is avoidance, by staying inside during peak sunlight hours or wearing protective clothing [6,137,138]. Since these two methods are not always an option, various topically applied photoprotectants, sunscreens, have been and are being developed.

Early sunscreens were designed to screen only UVB radiation, since the objective was to prevent edema and skin cancer, both of which effects are primarily caused by UVB [15,25-28]. Organic UVB protecting sunscreens typically consist of an aromatic core with two functional groups, one of which readily donates an electron and the other which readily accepts electrons, which allow for the dissipation of absorbed UVB energy through delocalization of charge across the molecule [6]. The most commonly used UVB protecting sunscreens belong to four broad categories of chemicals which are derivatives of *p*-aminobenzoic acids (PABA), octocrylene, salicylates and cinnamates as shown in figure 2.7 [6].

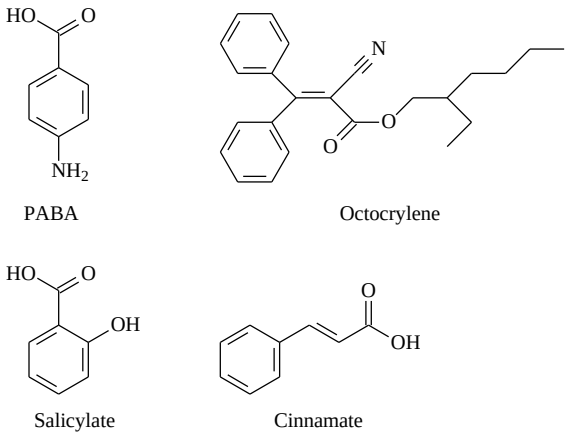


Figure 2.7 – UVB Absorbing Sunscreens

The PABAs are exemplified by such compounds as ethylhexyl dimethyl PABA and ethyl dihydroxypropyl PABA and the salicylates by such compounds as ethylhexyl salicylate and ethyl triazone [6,139]. Cinnamate derivatives include ethylhexyl methoxycinnamate and octylmethoxycinnamate, while octocrylene is often used unmodified [6,139,140]. These early sunscreens presented a number of problems. PABA, one of the first sunscreens introduced in the 1920's, proved to induce photocontact allergies and was implicated in causing autoimmune diseases [6].

As mentioned, these sunscreens were designed primarily to protect against the effects of UVB radiation. The ability of the compounds to protect against UVB damage is rated by the sun protection factor (SPF) which is an indication of how many times longer it takes to produce edema compared to unprotected skin. For example, if under given light conditions, sunburn is produced in 1 min, wearing an SPF 15 sunscreen would result in a delay of 15 min before sunburn would occur [138]. However, as it became clear that that the UVA portion of sunlight could cause health effects such as photoaging [15] and could contribute to skin cancer [8,34], the need for broader spectrum sunscreens that protected from both UVA and UVB became clear [6].

The first of the new broad spectrum sunscreens, oxybenzone (benzophenone-3) (Fig 2.8), appeared in the 1980's [6]. However, oxybenzone has been shown to be absorbed by the skin and may have estrogenic activities [6]. Since then a new generation of broad spectrum sunscreens have been introduced. These include butyl methoxydibenzoylmethane (avobenzone, Parsol 1789), drometrizole trisiloxane (Mexoryl XL), and terephthalidene dicamphor sulfonic acid (Mexoryl SX), as shown in Figure 2.8

[137-139]. Currently, no universal rating system such as SPF exists for UVA protection though such a system has been proposed [138].

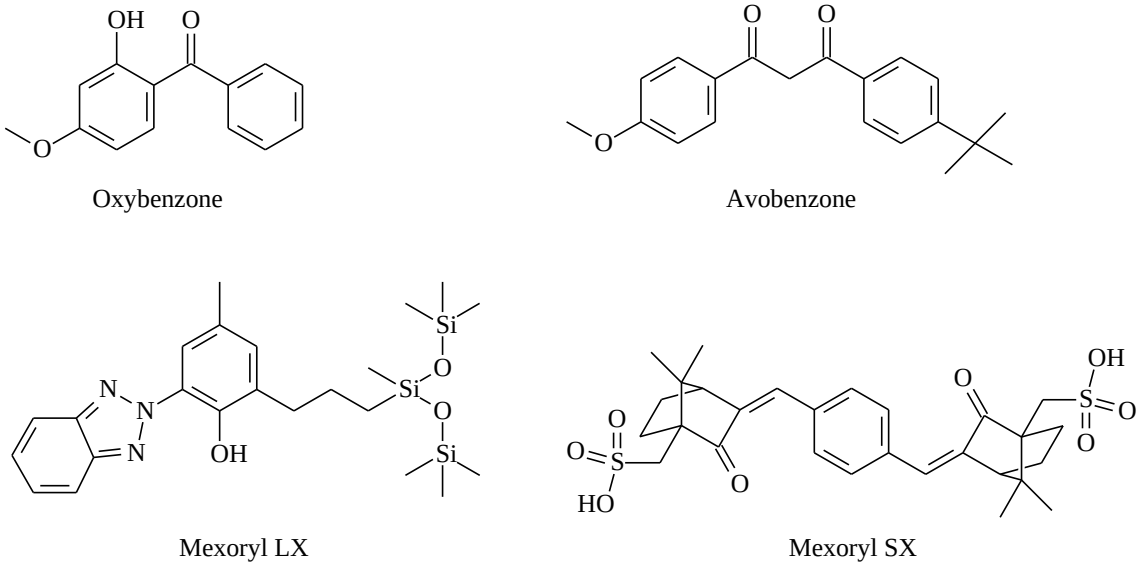


Figure 2.8 – Broad Spectrum Sunscreens

The efficacy of the various sunscreens currently on the market against skin cancer and photoaging is not entirely clear. Using human subjects and solar simulated radiation (SSR) over 4 days, sunscreens containing only methoxycinnamate have proven effective in the prevention of thymine dimer formation, though other DNA damage was not assessed [13]. Similar positive results were seen in human subjects in a long term study over two years. In this case use of a sunscreen containing a mixture of oxybenzone, octyl methoxycinnamate and octyl salicylate proved effective in slowing the rate of accumulation of actinic keratoses (AKs), a pre-cancerous lesion [141]. Another combination sunscreen containing 2-ethylhexyl *p*-methoxycimmamate, oxybenzone and octyl salicylate with an SPF rating of 17 was shown to decrease AKs in humans [142]. Mexoryl SX has also proven to be an effective sunscreen. In a study using mice treated topically with Mexoryl SX and exposure to SSR resulted in a longer tumour latency

period and was determined to have a tumour protection factor of 2.4, compared to 1.3 for cinnamates [143]. Mexoryl SX has also been shown to decrease DNA fragmentation in cultured keratinocytes as measured by the Comet assay, as well as preventing the accumulation of p53, a marker of DNA damage [137].

Although sunscreens appear to be effective against skin cancer, the controversy arises over the stability of these compounds when exposed to UVR. Testing of a sunscreen containing octyl methoxycinnamate, oxybenzone and Parsol 1789 showed that the Parsol 1789 and octyl methoxycinnamate were both unstable, breaking down into smaller non-functional molecules and losing their ability to absorb UVR upon exposure to light [144]. Similar results were seen with other combinations of sunscreens. When exposed to SSR, a sunscreen containing octyl methoxycinnamate, oxybenzone and octocrylene proved the most stable followed by a formulation containing octyl methoxycinnamate, Parsol 1789 and octylcrylene, then a combination of octyl methoxycinnamate, oxybenzone and octyl salicylate and finally a relatively unstable mixture of Parsol 1789 and 4-methylbenzilidene [145]. It was also found that the addition of octocrylene to any of the other tested compounds improved stability [145]. This possible lack of stability of sunscreens could result in a loss of photoprotection, and suggests that other new photoprotective compounds are needed.

2.4.2 Natural Product Based Sunscreens

Efforts to find more effective and/or photostable sunscreens have taken a number of paths, one of which is the investigation of natural products. Since plants are generally exposed to large amounts of ultraviolet radiation, many have evolved defences against

the harmful effects of light in the form of secondary metabolites, which may be exploited for human use.

A body of research exists investigating the photoprotective effects of α-tocopherol, or vitamin E. Vitamin E had been shown to prevent UV-induced skin damage by a combination of anti-oxidant and UVB absorptive methods as reviewed by Krol, et. al. [146]. Using shaved mice and UVB radiation, topical application of a cream containing as little as 1% (w/w) α-tocopherol has been shown to decrease the formation of thymine dimers by 43% compared to controls [147]. Further experiments with the same model showed that α-tocopherol was more effective in preventing thymine dimer formation than ethylhexyl salicylate and oxybenzone, and had similar efficacy to 5% octyl methoxycinnamate. However, in this experiment it appeared that absorbance of the α-tocopherol by the cell was necessary for the protective effects, suggesting involvement of an anti-oxidative mechanism [140]. Topical application of α-tocopherol has also been shown to decrease tumor formation. In mice treated topically with α-tocopherol for three weeks prior to UVB exposure, only 42% developed skin cancer 33 weeks post-UVB exposure compared to 88% of control mice [148].

Comparison of topical α-tocopherol *in vivo* to the related compounds γ-tocopherol, δ-tocopherol showed that all three compounds reduced the formation of thymine dimers [140]. The same study showed that α-tocopherol acetate and α-tocopherol methyl ether did not affect thymine dimer formation [140]. This study also found that optimal prevention of thymine dimer formation required uptake of the tocopherols into the cell [140].

Besides vitamin E, a number of plant extracts have been tested for photoprotective properties. The isoflavones have shown some positive effects in preventing both skin cancer and photoaging. Topical application of genistein (4',5'7-trihydroxyisoflavone) to mice 10min to 24hr prior to UVB exposure showed that this isoflavone was effective in preventing induction of kinases leading to production of MMP-1, prevented oxidation of LDL, reduced both thymine dimer formation and oxidative DNA damage by 90% and prevented sunburn [149]. The inhibition by genistein of UVR induced enzyme cascades leading to photoaging has also been shown to occur in human skin *in vivo* [150]. Another isoflavone 2',4',7-trihydroxyisoflavone has also been shown to reduce MMP-1 induction at both the RNA and protein levels in human fibroblasts [151].

Other polyphenols that have been investigated include the flavone apigenin, green tea extracts, and cyanidin-3-*O*-glucoside. Apigenin has been shown to reduce the incidence of cancer by 52% when applied topically to mice 24 hours prior to UVA/B radiation [152]. The green tea polyphenols, particularly (-)-epigallocatechin-3-gallate (EGCG) have also be tested as topical photoprotectants and have been found to prevent the depletion of the anti-oxidant enzymes glutathione peroxidase and catalase as well as GSH levels. EGCG also decreased UVB initiated lipid and protein oxidation and inhibited phosphorylation of ERK, ANK and p38. These data seem to suggest that EGCG acts primarily through an anti-oxidant mechanism [153]. Finally, pre-treatment of HaCaT cells with cyanidin-3-*O*-glucoside has been shown to decrease UVB induced IL-8, procaspase-3 and AP-1 induction [154]. A less related compound, the lignan melanocin A has been shown to reduce MMP-9 induction when used to pre-treat HaCaT cells prior to UVB exposure [155]. This same compound has also been shown to reduce wrinkling

as well as MMP-2 and MMP-9 induction in mice when used as a topical photoprotectant [155].

In conclusion, various naturally occurring vitamins and polyphenols have shown potential for use as topical, broad spectrum sunscreens. However, methods of protection remain to be elucidated and a large number of compounds still need to be investigated to find the best option.

2.4.3 Summary

There are currently a variety of commercial sunscreens available to protect skin from UVR related damage, including the older UVB screens and newer broad-spectrum screens. The older generation of sunscreens, such as PABA derivatives and cinnamates, effectively screen UVB radiation but do not protect from what is now recognized as the equally harmful UVA radiation. Newer sunscreens such as Oxybenzone and mexoryl SX protect from both UVA and UVB radiation, but their efficacy and photostability are not clear.

In efforts to find more effective sunscreens, a number of plant derived natural products have been investigated. Such compounds as α-tocopherol, various polyphenols and their sugar derivatives have shown some effectiveness, non-have been marketed to date. One naturally occurring compound that may have potential as a topical sunscreen is the flavanol quercetin, which will be discussed in the next section.

2.5 Quercetin

2.5.1 Chemistry of Quercetin

2.5.1.1 Physical Properties of Quercetin

Quercetin (3, 3', 4', 5, 7-Pentahydroxyflavone) (Figure 2.9) is a naturally occurring polyphenol produced by a variety of plant species [156,157]. It is part of a group of phytochemicals called polyphenolic flavanoids, which are characterized by a diphenylpropane ($C_6C_3C_6$) skeleton. Specifically, quercetin is part of a sub-group called flavanols which are characterized by a hydroxy group at the 3 carbon, and a double bond between carbons 2 and 3 [156].

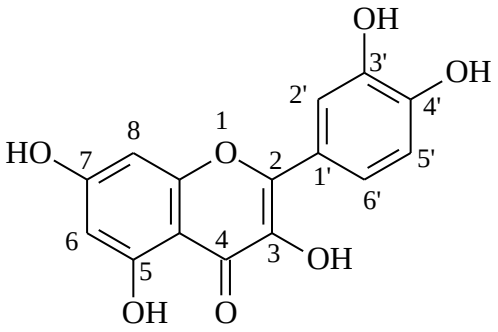


Figure 2.9 - Structure of Quercetin

Quercetin is a solid at room temperature which has a yellow colour in the visible spectrum and two strong absorbance bands in the ultraviolet [158]. The maximal UV absorbance bands (Figure 2.10) of quercetin occur at 256nm and 368nm with molar absorptivity values of 28300 $M^{-1}cm^{-1}$ and 28400 $M^{-1}cm^{-1}$ respectively [159].

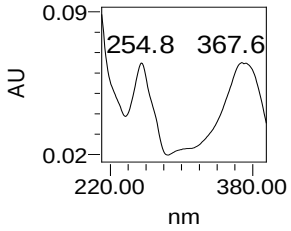


Figure 2.10 UV Spectra of Quercetin in Methanol

Quercetin alone in solution has no fluorescence chromophore, however complexation with metals such as Al^{3+} [160,161] or proteins such as albumin [162] results in a strong fluorescence chromophore with absorbance at 430nm and emission at 500nm.

Quercetin is only sparingly soluble in water but readily soluble in alcohols (1g/290mL) as well as soluble in acetic acid and alkaline aqueous solutions [158]. Orally administered quercetin has a low toxicity with an LD_{50} of 160mg/kg in mice [158]. In cultured human cells, quercetin has an LD_{50} of 300µM [163].

2.5.1.2 Oxidation of Quercetin

Due to its low pK_a values (6.74, 9.02 and 11.55) which render it negatively charged at neutral pH as well as a high redox potential, quercetin is susceptible to oxidative process [164]. As a result, the oxidation products of quercetin are well described in the literature [165,166]. A recent article by Zhou reported over 20 oxidation products for quercetin [166].

Some of the early work on the oxidation of quercetin was conducted by Westlake et. al. working with microbial decomposition of rutin [165]. Three water soluble products were found which were identified as protocatechuic acid (3,4-Dihydroxybenzoic Acid), phloroglucinol carboxylic acid (2,4,6-Trihydroxybenzoic Acid) and a phloroglucinol carboxylic acid - protocatechuic acid ester (quercetin depside) [165], as shown in figure 2.11.

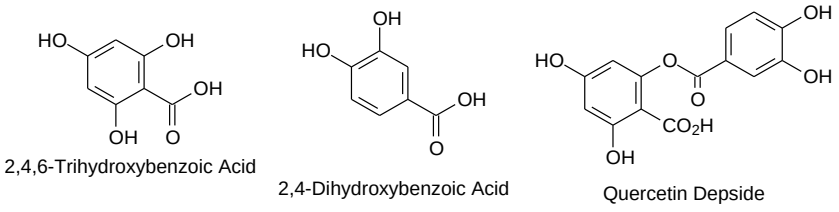


Figure 2.11 - Bacterial oxidation products of quercetin [165]

The compounds shown in figure 2.11 were later generated from quercetin by photosensitized oxygenation of quercetin using rose Bengal to generate singlet oxygen [167]. The depside was proposed to be formed by addition of singlet oxygen to quercetin to form the oxohydroperoxide followed by the loss of carbon monoxide through either a five or four membered cyclic oxoperoxide [167,168]. Experiments using ^{18}O labelling later showed that both base and enzymatically catalyzed oxygenation of quercetin yielded the same products, and that the reaction did in fact proceed through the five membered cyclic oxoperoxide with the loss of carbon monoxide [169]. Mechanistic studies using the copper dependant bacterial enzyme quercetin 2,3-dioxygenase also showed that the formation of depside proceeded by 1,3-cycloaddition rather than 1,2-cycloadditon, showing that the five membered cyclic oxoperoxide was the correct intermediate, and that the copper ion acts as an oxidant towards quercetin [170]. The proposed reaction mechanism is shown in Figure 2.12.

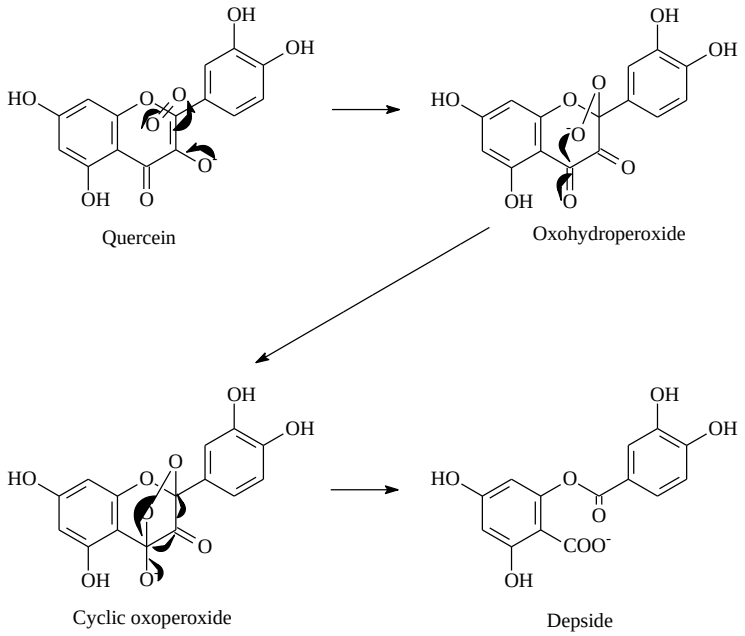


Figure 2.12 - Proposed mechanism for oxygenation of quercetin [169]

Other studies of the enzymatic oxidation of quercetin have used peroxidase and H_2O_2 [171]. This process yielded more than 20 compounds of which were identified 3,4-dihydroxybenzoic acid; 2,4,6-trihydroxybenzoic acid; methyl 2,4,6-trihydroxyphenylglyoxylate, 2-(3,4-benzochinoyl 3,5,7-trihydroxy-4H-1-benzopyran-4-one; 2-(3,4-dihydroxyphenyl)-2-hydro-3,5,7-trihydroxy-3-methoxy-4H-1-benzopyran-4-one; 2,3-epoxy-2-(3,4-dihydroxyphenyl)-3-[4O-[2-(3-hydroxyphenyl)-3,5,7-]trihydroxy-4H-1-benzopyran-4-only]-5,7-dihydroxy-4H-1-benzopyran-4-one; and a trimer related to the last product [171].

Oxidation of quercetin by bubbling air through a 70% ethanol in water solution of quercetin produced three main oxidation products. These products were identified as 2,4,6-benzenetriol, 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid [172]. The authors propose that formation of the two benzoic acids proceeds through formation and subsequent cleavage of quercetin depside as seen in other quercetin oxidation reactions. However, the authors propose that the accepted mechanism of depside formation, namely oxidative decarbonylation, is unlikely to proceed under their mild conditions and that oxidative decarboxylation is a more likely process, proceeding from the keto-tautomer [172].

A similar experiment used horseradish peroxidase and H_2O_2 treatment to generate quercetin oxidation products also yielded at least 20 oxidation products [173]. However, the experiment was also conducted with the addition of glutathione as a radical scavenger. The addition of GSH resulted in the production of only two products which were identified as the 6-glutathionylquercetin and 8-glutathionylquercetin, with the

adducts forming on the A ring. The authors propose a mechanism wherein the quercetin semi-quinone is oxidized to the *o*-quinone which isomerizes to the *p*-quinone methide which has electrophilic character at the C6 and C8 positions [173]. A comparison of the GSH adducts formed upon oxidation of several structurally related compounds supported this mechanism, as it showed that only those compounds containing a catechol group on the B-ring, and therefore capable of forming the *o*- and *p*-quinone, showed substitution of GSH on the A ring [174]. Similar results were seen when tyrosinase was used to generate the unstable quercetin *o*-quinone, with 6- and 8-gluathionylquercetin being formed. In addition, these adducts were shown to be unstable and capable of inter-conversion [175]. The position of GSH adduct formation has also been shown to be pH dependant. At pH 7.0, the C6 and C8 adducts described above form, but as pH increases the amount of C6 and C8 adducts decreases until at pH $\geq$ 9.5, they are completely replaced by 2',5'-digluathionyl quercetin and 2',5',6'-triglutathionyl quercetin. In contrast, at pH < 7 two products are formed, both of which have added one GSH and one H_2O. The authors attribute the addition of GSH to the B ring under alkali conditions to the loss of the proton at the C3 hydroxyl, preventing the formation of the quinone methide [176].

Studies comparing GSH binding to quercetin oxidation products with that of other cellular thiols have shown that GSH is not necessarily the favoured scavenger. When GSH, cysteine and *N*-acetylcysteine were used the favoured conjugates were 6- and 8-cysteinylquercetin. However, since GSH is present in much higher concentrations in the cell than cysteine or *N*-acetylcysteine, the GSH adducts are likely still the favoured conjugates *in vivo* [177].

Electrochemical oxidation studies with quercetin have shown that there are three distinct oxidation events which can occur at pH 7.0. The first oxidation at 150mV occurs at the catechol group on the B ring, the second at 500mV corresponds to oxidation of the hydroxyl group at C3, and the final oxidation occurs at the 5,7-dihydroxy substiuent on the A ring [178]. This study identified 2 oxidation products, distinct from the expected 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid, but the products were not identified [178]. In contrast a later electrochemical oxidation study of quercetin showed the production of 18 different products. The expected 3,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid were present along with their esters as were the depside and its ester and 2,4,6-benzenetriol. Other products included 3,4-dihydroxy- and 2,4,6-trihydroxy-phenylglyoxylic acid, and taxifolin, the C2-C3 saturated form of quercetin. Also present were 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-benzofuran-3-one, in which the C ring has become a furan, quercetin chalcone (3-(3,4-dihydroxy-phenyl)-2-hydroxy-1-(2,4,6-trihydroxy-phenyl)-propenone, resulting from cleavage of the 1,2 heteroatomic bond in the C ring, as well as 2-(3,4-dihydroxyphenyl)-2-(hydroxyl(ethoxy)-5,7 - dihydroxy-chroman-3,4-dione and 2-[carboxy-(3,4-dihydroxyphenyl)-hydroxy-methoxy]-4,6-dihydroxybenzoic acid. Finally a dimer that had formed between the catechol moiety and the C2-C3 double bond was detected [179]. In addition to the structural data, a number of possible mechanisms were proposed [179].

The formation of the quercetin dimer was also investigated using 2,3'-azobisisobutyronitrile (AIBN) as the oxidizing agent. This study showed that in addition to the C2-C3 double bond, a free C3 hydroxyl is essential for dimer formation [180].

Quercetin oxidation products generated using the radical generator AIBN produce similar products to those seen when electrochemical oxidation is used, although with less variety. The oxidation of quercetin by AIBN in acetonitrile produced four compounds. These included the benzofuran derivative 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxy-benzofuran-3-one and the depside. The authors proposed that rather than the depside being formed through the five membered cyclic oxoperoxide, both the depside and the benzofuran were formed through a common carbocation intermediate [181]. The other products found by AIBN oxidation were methyl 3,4-dihydroxyphenylglyoxylate and the quercetin dimer [181]. The products of lipid radical reactions with quercetin have also been studied and identified as the depside and the quercetin dimer [182]. Mechanistic studies of the chemical oxidation of quercetin in protic and aprotic solvents using diphenylpicrylhydrazyl and ceric ammonium nitrate as radical generators as well as under auto-oxidative conditions have also been conducted. These studies have shown that the first step in the reaction following hydrogen abstraction to form the radical species is rapid disproportionation to form the *o*-quinone which rapidely establishes an equilibrium with the quinone methide [183].

The oxidation of quercetin and the mechanisms which result in the observed products are complex and seem to be somewhat dependant on the oxidant species and the solvent as well as the presence of radical scavengers. As a result the mechanisms involved in the formation of the oxidation products are still a matter of some debate. However, even if the mechanisms involved are not fully known, the resulting oxidation products from a wide variety of oxidation methods are fairly well characterized and have been summarized by Zhou [166].

2.5.1.3 Photochemistry of Quercetin

Due to the strong absorbance of quercetin in the UVR range [159] the question of quercetin's photochemistry is pertinent. Work has been done which showed that quercetin in an ethanol solution disappeared within 18 to 37 hours, as monitored by the UV absorption bands when exposed to UVC radiation [184]. Other studies on the stability of quercetin in alcohol solution, in this case methanol, found that quercetin absorbance decreased by 17% after 15 hours of combined UVA and UVB radiation [185]. In both of these experiments, air was bubbled through the alcohol solutions, raising the possibility of photoinduced singlet oxygen resulting in an oxidative decomposition [184,185]. In contrast, quercetin appeared to be resistant to photolysis when dissolved in 1:1 benzene:isopropanol and exposed to a mercury lamp which the authors suggested could be the result of tautomerization and deprotonation of the excited state or intramolecular quenching, all of which would increase photostability [186].

Exposure of quercetin deposited on cellulose to combined UVA/UVB radiation also resulted in photobleaching, but the extent of this effect was not quantitated [185]. Exposure of wool dyed with quercetin to combined UVA/UVB radiation also showed decomposition of quercetin , with 3,4-dihydroxybenzoic acid being identified as a degradation product, presumably being formed through the depside. The expected 2,4,6-trihydroxybenzoic acid was not observed, though this was attributed to loss during an acid extraction process [187].

2.5.2 Dietary Intake of Quercetin

2.5.2.1 Sources of Quercetin

Quercetin is found in many members of the plant kingdom, including many edible species, and the estimated daily intake of quercetin for North Americans is 25 mg/day

[188]. Quercetin is found in high levels in onion, lettuce, broccoli, cranberries and apple skins [156]. Quercetin, along with other flavanoids, is also found in a number of common beverages including tea, beer and wine [188].

Quercetin is most commonly found in plants, and ingested, as a glycoside with the linkage to the sugar occurring through the hydroxyl group at the C3 position to form quercetin-3-O-glycoside [188]. The most commonly consumed quercetin glycosides are rutin (quercetin bound to rutinose) and quercetrin (quercetin bound to rhamnose) [188].

2.5.2.2 Absorption of Quercetin

Since most dietary flavanoids are ingested as the glycosides, absorption of quercetin necessarily involves absorption of the quercetin glycosides. Due to their high molecular weight and their generally hydrophilic nature, ingested quercetin glycosides largely pass unaltered through the small intestine [188], with little or no absorption of the glycoside occurring at this point [189]. However, it has been shown in humans that rutin is absorbed in the distal small intestine or colon [190,191]. The literature further demonstrates that in the lower small intestine and colon, the quercetin glycosides are hydrolysed prior to absorption and that it is in fact the aglycone form of quercetin which is absorbed [188,191]. This cleavage of the sugar group appears to be primarily caused by glycosidases produced by intestinal microflora [188]. However, there is also some evidence that some flavanoid glycosides can be hydrolysed in the brush border of mammalian small intestine by lactase phloridzin hydrolase, following which the aglycone can be absorbed [189]. There is also some evidence for transport of rutin quercetin-3-glucoside across the jejunum following infusion of isolated rat small intestine when gut microflora were not present [192].

Although quercetin in plants is mainly encountered in a glycated form, a small amount of aglycone may be present [188]. Also, quercetin aglycone is available as a dietary supplement from a number of manufacturers [157,188]. In contrast to the glycosides of quercetin, quercetin aglycone is absorbed in the upper portion of the duodenum [191]. Quercetin is lipophilic enough in character that absorption does not require active transport, and absorption in humans is in the range of 36 to 54% [189]. Once in the circulatory system, quercetin is extensively protein bound with 99.1% protein binding being reported in humans [189,191,193]. In addition, it has been shown that topically applied quercetin can effectively cross the skin with 50% of the dose appearing in the dermis after 12 hours [194].

2.5.2.3 Quercetin Metabolism

Although quercetin aglycone is absorbed from the intestine following oral dosing with quercetin aglycone or a quercetin glycone such as rutin, little or no free quercetin is found in the blood [189]. This is due to quercetin's high degree of plasma protein binding (99.1%) [189,191,193] and rapid metabolism.

Due to the presence of 5 hydroxyl groups on quercetin, the cytochrome P450, or CYP enzymes do not act upon it and no CYP mediated metabolism of quercetin is observed *in vivo* [195,196]. In contrast, the quercetin related flavanoids kaempferol, which lacks the 3' hydroxyl, and tamarixetin, which has a 4' methoxy group instead of a hydroxyl, are metabolized to quercetin by CYPs 1A2, 3A4 and 2C9 [195].

Even though quercetin is not metabolized by the cytochrome P450's, quercetin does have effects on some P450 enzymes. In human liver microsome samples, quercetin, along with other flavanoids, inhibited CYP 1A [197]. Studies using galangin, which

lacks the catechol moiety, have shown that it is hydroxylation at the 3 and 5 positions that is important for this inhibition [198]. Quercetin also showed a biphasic activity for CYP 3A4 and CYP 3A3, activating these enzymes at low concentrations and inhibiting their activity at higher concentrations [197]. In addition quercetin has been shown to be an efficient inhibitor of Cyp 2B1 from rat microsomes, showing greater inhibition than the related compounds catechin, epigallo-catechin gallate, diosmin, and naringenin [199].

The abundance of hydroxyl groups on quercetin which make it resistant to phase I metabolism also makes it highly susceptible to phase II conjugation. Free quercetin is not found in the blood in either rat or human subjects, but a large number of quercetin conjugates are found in plasma and urine [189,190,200,201]. The major quercetin metabolite found in urine following intraperitoneal administration of quercetin to rats is 3'-O-methylquercetin, the formation of which appears *in vivo* to prevent the carcinogenic potential of quercetin seen *in vitro* [200]. An analysis of human urine following the consumption of cooked onions showed a large number of metabolites including three quercetin-diglucuronides, methyl-quercetin-diglucuronide, two quercetin-monoglucuronides, two quercetin-glucoside sulphates, quercetin-monoglucuronide sulphate, four methyl-quercetin-monoglucuronides and methyl-quercetin [201].

In vitro cell culture studies of quercetin metabolism have also been performed, and have yielded more detailed metabolite structures. Treatment of mouse melanoma cells with quercetin resulted in the production of two glutathione adducts with addition occurring at the 6 and 8 positions of quercetin, with addition the 8 position occurring at a higher yield [202].

Treatment of rat microsomes from the liver and small intestine with quercetin produced a number of sulphonated, methylated and glucuronidated metabolites. In rat liver, 6% of the administered quercetin was methylated (65% at the 3' position, 35% at the 4'), 14% was sulphonated (12% at the 7, 88% at the 3') and 18% was glucuronidated (40% at 7, 42% at 3', 18% at 4'). In rat small intestine microsomes, 4% was methylated (67% at 3', 33% at 4'), 25% was glucuronidated (9% at 3, 41% at 7, 46% at 3' and 4% at the 4'). No sulphonation products were found in the small intestine microsomes [203]. The same experiment using human liver and intestinal microsomes showed similar results with some differences. Human liver microsomes produced 5% methylation (66% at 3', 34% at the 4'), 22% glucuronidation (13% at 3, 65% at 7, 19% at 3' and 3% at 4'). Surprisingly, human liver microsomes, like rat intestinal microsomes, produced no sulphonate conjugates of quercetin. Human intestinal microsomes produced the greatest variety of quercetin conjugates with 1% methylation (62% at 3' and 38% at 4'), 5% sulphonation (12% at 7 and 88% at 3'), and 61% glucuronidation (20% at 3, 4% at 7, 49% at 3' and 27% at 4') [203].

Treatment of human fibroblast cultures with quercetin resulted in the formation of two metabolites, a 2' glutathione-quercetin conjugate and a quercetin quinone/quinone methide – glutathione conjugate [204]. The same study showed that treatment of fibroblasts with 3'-O-methyl quercetin or 4'-O-methyl quercetin resulted in formation of similar products, suggesting that demethylation of these compounds is the initial reaction [204].

2.5.3 Biological Effects of Quercetin

2.5.3.1 Antioxidant Properties of Quercetin

As discussed briefly in section 2.5.1.2, as a result of the abundance of hydroxyl groups on quercetin, the low pK_a values and high redox potentials quercetin is oxidatively labile [164]. Quercetin rapidly reacts with radicals, such as the azide radical, to form a quercetin radical species with a rate constant $6.6x10^{-9}$ $dm^3mol^{-1}s^{-1}$ [79]. The resulting radical species is quite stable with a decay rate constant of $3.4x10^{-6}$ $dm^3mol^{-1}s^{-1}$[79]. These properties make quercetin an effective anti-oxidant, and the anti-oxidative properties of quercetin have been extensively studied.

A variety of methods have been used to determine the anti-oxidant capacity of quercetin. Using the Trolox Equivalent Antioxidant Capacity (TEAC) test which measures the ability of a compound to scavenge the $ABTS^{\cdot+}$ radical, 1mM quercetin has been found to be equivalent to 4.7mM of the vitamin E derivative Trolox [156]. This value makes quercetin among the most effective antioxidants found among the polyphenols found in tea extracts [205]. However, another study using the same method found an equivalency of only 2.5 [206]. These values are determined over longer term exposure of quercetin to the radical species, but an assessment of the fast reaction kinetics results in different numbers, with Trolox equivalences of 1.01 and 1.21 being determined for the reaction at 0.1 sec and 2 sec respectively [207].

Another system which has been used to determine the anti-oxidant capacity of quercetin is the scavenging of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radicals, which resulted in a stoichiometry of 2.35 radicals per quercetin molecule in dimethylformamide and 3.19 in methanol [183]. In addition, quercetin has been shown to scavenge 3.7 Fe^{3+} ions reduced per molecule for the Ferric Reducing Antioxidant Power assay (FRAP)

[206]. Finally quercetin has been found to have an IC_{50} of 0.154µM against peroxynitrite oxidation of dihydrorhodamine [208].

Quercetin has also been shown to be an effective scavenger of a variety of reactive oxygen species including hydrogen peroxide [174], and superoxide [209]. The rate constants for these, and other scavenging reactions, have been determined to be $^{\cdot}OH = 43x10^{8}M^{-1}s^{-1}$, *t*-butyloxide = $25x10^{8}M^{-1}s^{-1}$, lipid peroxide = $0.18x10^{8}M^{-1}s^{-1}$ and superoxide = $0.0009x10^{8}M^{-1}s^{-1}$ [209].

From a biological point of view, the most significant anti-oxidant effect of quercetin may be the prevention of lipid peroxidation, and a number of studies have looked at this property with varying results. Looking at the formation of malondialdehyde (MDA) formation initiated by Fe^{2+} and using the thiobarbituric acid reactive substances (TBARS) method to measure oxidation of flax oil, quercetin was determined to have an IC_{50} of 1.7µM [210]. This is similar, though lower than the IC_{50} determined by TBARS in the auto-oxidation of rat cerebral membranes where a value of 3.09µM was determined [211], and both these values were much lower than TBARS IC_{50} of 17.6µM determined for $FeSO_4$ peroxidation of microsomes [212]. Other methods of determining IC_{50} values for quercetin vary even more widely. Using conjugated diene formation as a measure of iron initiated linolate peroxidation has given an IC_{50} = 28.61µM [211], measurement of *t*-butyl-hydroperoxide initiated chemiluminesence of rat liver homogenates gave an IC_{50} of 200µM [213].

Stoichiometricly, quercetin has been found to protect flax oil against iron oxidation by chelating 3 molecules of Fe^{2+} per molecule of quercetin [210]. This value is comparable

to the stoichiometry of 3.5 radicals trapped per quercetin molecule of quercetin when preventing AMVN-catalyzed oxidation of methyl linolate [214].

Minimal effective doses of quercetin for the protection of lipids from oxidative damage have also been determined. Quercetin protection of lipoproteins from oxidation by the radical generator AAPH required a minimum concentration of 2μM to be effective [215]. This value is comparable to the value of 5μM needed to prevent peroxidation of low density lipoprotein (LDL) caused by copper ions [216]. However, both these values differ greatly from the minimum effective dose of 82.1μM quercetin found necessary to scavenge the alkylperoxyl radical products of lipid peroxidation [217].

In more complete biological systems, quercetin has also been found to be an effective antioxidant. In skin cells, when GSH synthesis is inhibited by buthione sulfoximine, depletion of GSH leads to the accumulation of peroxides in the cell which lead to cell death. Treatment of cultured fibroblasts with quercetin, at an EC_{50} of 30 to 40μM, significantly reduced the level of cell death, while not altering the GSH levels. This suggests that quercetin was effectively taking up the radical scavenging duties of the depleted GSH [218]. Similar results were also found in cultured nerve cells, where pre-treatment with a minimal effective dose of 10μM quercetin protected the cells from H_2O_2 induced neurodegeneration [219].

The large variation seen in the IC_{50}, stoichiometry, and minimal effective dose numbers found for quercetin in the literature is a cause of some concern. However, these differences may be explained by the fact that the studies use a variety of endpoints, oxidation initiators, and reaction media to determine the numbers. As a result the anti-

oxidant mechanism, availability of quercetin and degree of incorporation of quercetin into membranes may all affect the anti-oxidant values.

A comparison of the anti-oxidant capacity of quercetin to that of other flavonoids has shown mixed results. In a study of the anti-oxidant effects of various polyphenols against Cu^{2+} induced damage to LDL, quercetin proved to the most effective of the compounds tested [220]. The authors concluded that quercetin has a large number of reactive centres and as such is more reactive to reactive oxygen species than other compounds like the quercetin glycoside rutin [221]. It is also supported by the fact that quercetin has a higher TEAC value than most flavanoids and their glycosides [156]. In contrast, quercetin was found to be less effective than rutin in protecting linoleic acid from auto-oxidation, perhaps because of differences in solubility [222].

The comparison of quercetin to other flavanoids in terms of anti-oxidant capacity has led to some conclusions about the structural factors affecting this ability. The structural components of the flavanoids which increase anti-oxidative capacity appear to be the catechol group on the B-ring, the double bond between carbons 2 and 3, and the presence of a 3 and 5 hydroxyl on the A ring along with the C-4 oxo-group, all of which quercetin has [156]. These structural features allow quercetin to suppress lipid peroxidation at three points in the oxidative process, the formation of superoxide radicals which can be scavenged, the generation of hydroxyl radicals and the formation of lipid peroxyl radicals [223].

Although quercetin is primarily thought of as an anti-oxidant, it can act as a pro-oxidant as well. In rat liver microsomes, at concentrations above 100μM, quercetin has been shown to increase the generation of hydroxyl radicals from H_2O_2 in the presence of

Fe^{3+}-EDTA by eight fold. However, this effect was not seen with Fe^{3+}-citrate or Fe^{3+}-ADP in the presence of H_2O_2 [224]. There is some evidence that this pro-oxidant effect of quercetin occurs through the *o*-quinone or semi-quinone product of quercetin oxidation which could result in the formation of superoxide or depletion of cellular GSH [225].

2.5.3.2 Enzyme Related Effects of Quercetin

In addition to the anti-oxidant properties of quercetin, there are a number of other biological effects as well. In addition to its anti-oxidant capacity, quercetin can affect the cell's own defences against oxidative stress [226,227]. Quercetin can also modify the activity of a number of CYP enzymes as discussed in section 4.2.3 [197-199]. Finally, quercetin can modify a number of enzyme cascade pathways [228-230].

In female rats, quercetin has been shown to reduce the activity of glutathione reductase, catalase and glutathione peroxidase in red blood cells while still reducing the overall oxidative stress, suggesting that a feedback mechanism exists which down regulates the expression of anti-oxidant enzymes when quercetin is present [226]. In contrast to this down regulation of anti-oxidant enzymes, quercetin has also been found to increase synthesis of γ-glutamylcysteine transferase, an important enzyme in the synthesis of glutathione, resulting in an increase in cellular glutathione levels [227].

In the human endothelial cell line ECV304 quercetin has been shown to down regulate TNF-α induced ICAM-1 expression in a dose dependant manner with significant deceases seen from 1 to 50 μM quercetin. This down regulation seems to be controlled through the inhibition of AP-1 activation and inhibition of the JNK pathway [228]. This inhibition of ICAM-1 expression leads to a decrease in inflammation, but inhibition of AP-1 may also be tumor promoting as it inhibits apoptosis [112,231,232].

Quercetin has also been shown to affect ERK, though what those effects are remains unclear. In cultured neuronal cells, quercetin and its methylated metabolites have been shown to inhibit the pro-survival signal enzymes ERK and Akt/PKB which leads to an induction of neuronal cell death through increased activity of caspase-3 in a dose and time dependant manner with effects seen from 10 to 30uM quercetin [233]. In contrast, in mouse macrophage cells, 25, 50 and 100µM quercetin has been shown to induce phosphorylation of ERK, but not JNK. This increase in ERK activity induces heme-oxygenase-1 activity at 50µM quercetin which protected against H_2O_2 induced apoptosis [229]. In addition, in myoblast cells treated with 10 to 20µM quercetin for 1 hour, or with 1 and 3 µM quercetin for 18 hours gave significant protection from H_2O_2 [230]. This protection appeared to occur through increased phosphorylation of the anti-apoptotic proteins Akt and ERK1/2 [230].

Finally, Nagata et. al showed that treatment of cultured melanocytes with 5µM, 10µM or 20µM quercetin for up to seven days resulted in an increased production of melanin in the absence of UV light, through activation, but not induction, of tyrosinase [234]. This same group also showed that this effect occurs in a reconstructed three-dimensional epidermal model when quercetin was added to the culture media [235]. These results suggest that quercetin may protect against UVR damage by a number of mechanisms.

2.5.3.3 Photoprotection by Quercetin

As previously stated in section 4.2, quercetin is produced by a wide variety of plant species, usually as a glucoside [156,188]. In many of these species, quercetin is upregulated in repose to UV radiation. In the leaves of *Brassica napu*s (canola) plants three quercetin glucosides and three kaempferol (no 3' hydroxyl, only a 4') glucosides

were upregulated in response to UVB radiation [236]. These were identified as quercetin-3-*O*-sophoroside, quercetin-3-*O*-sophoroside-7-*O*-glucoside, quercetin-3-*O*-sophoroside-7-*O*-glucoside-caffeoyl ester, kaempferol-3-*O*-sophoroside, kaempferol-3-*O*-sophoroside-7-*O*-glucoside and kaempferol-3-*O*-sophoroside-7-*O*-glucoside-coumaroyl ester [236]. This increase in flavanoids correlated with increased UVB protection in the form of a decreased degradation of the D1 protein of photosystem II [237].

Using wild type and transgenic strains *Petunia axillaris*, Ryan, et. al. found that exposure to UVB resulted in an increase in total flavonol content in the leaves, and in particular an increase in the quercetin derivative to kaempferol derivative ratio compared to non-UVB exposed plants. The change in ratio and the fact that increased anthocyanin content did not appear to be protective suggests that it is the quercetin derivatives that are most photoprotective [238]. This same group also showed that plants with an increased quercetin to kaempferol ratio due to UVB exposure had faster growth rate compared to those mutants which did not produce quercetin in response to UVB, further suggesting a photoprotective role for quercetin [239].

Similar responses have been seen in the skin of apples, *Malus domestica*. In a comparison of the skins of two cultivars of apple fruit it was shown that Granny Smith apples did not increase the levels of quercetin glycosides in sun exposed fruit while Braeburn apples showed a significant increase in quercetin glycosides in sun exposed portions of fruit skins [240]. The same study found that this increase in quercetin glycosides in Braeburn skin resulted in less UVB induced damage to the apples photosynthetic apparatus [240]. Another study in the Antonovka cultivar of apples found

that in shaded apples cuticular phenolics were responsible for UVA/UVB absorption, while in sun exposed apples, UVA absorption was primarily due to quercetin glycosides in the vacuoles. From this the authors concluded that a build up of quercetin in cells just below the cuticle is a dominant factor in the apples long term adaptation to high sun levels [241]. However, the mechanism by which quercetin protects the plants is not clear.

The photoprotective effects of quercetin glycosides have prompted a number of investigations into possible photoprotective effects of quercetin in animals. Since skin cancer [8-10] and immunosuppression [15,113] are two of the most significant effects of UVR exposure, the ability of quercetin to protect against these effects has been investigated. Steernberg, et. al. reported that in SKH hairless mice exposed to UVR for up to 17 weeks and fed a diet containing 4% quercetin by weight, no affect on the onset or growth of non-melanomic skin carcinomas was observed [242,243]. However, the quercetin treatment did prevent the UVR induced contact hypersensitivity suppression seen in control mice, likely by preventing the decrease in epidermal Langerhan's cells seen in unprotected mice exposed to UVR [242,243].

Because of the strong antioxidant capacity of quercetin, the ability of quercetin to prevent UVR induced oxidative damage has also been assessed. Quercetin was found to inhibit the peroxidation of phosphatidylcholine liposomes by UVC radiation at an IC_{50} of 6.24μM. In the same study, to assess the use of quercetin topically, the authors found that less than 1% of topically applied quercetin crossed the epidermis of excised human skin in 24 hours [244]. Another study using hairless mice, but treating with UVB found

that topical quercetin prevented UVB induced myeloperoxidase activity, GSH depletion and proteinase secretion, all markers of oxidative stress [245].

Using Sprague-Dawley rats exposed to UVA and intraperitoneal injections rather than topical application, Inal, et. al. found that animals given of 50mg/kg quercetin had reduced levels of malondialdehyde (MDA), a marker of oxidative stress, compared to those rats treated with UVA alone while GSH levels were unchanged [246,247]. The same studies also found that UVA significantly reduced hepatic levels of superoxide dismutase and catalase, but that quercetin partially prevent this reduction [246,247]. Similar results were found when rats were fed 50mg/kg quercetin in their diets with significant decreases in MDA levels and prevention of UVA induced reduction of superoxide dismutase and catalase being observed [248].

Quercetin has also been shown to affect the induction of matrix metallo protease-1 (MMP-1), a marker of photoaging. Using human fibroblasts and the naturally occurring quercetin derivative quercetin-3O-β-D-(6"-feruloyl)-galactopyranoside, Moon, et. al. showed that UVB-induced expression of MMP-1 was reduced in a dose dependant manner by the flavonol glycoside [249]. This same group also showed that quercetin aglycone inhibited MMP-1 activity with an IC_{50} of 39.6μM and that in fibroblasts, quercetin inhibited the chemically induced production of MMP-1, likely through the inhibition ERK and MAPK activation [250].

Finally, quercetin has been shown to be effective in the prevention of pharmaceutical photosensitization. The NSAID ketoprofen is used as both a topical [251] and as a oral [252] analgesic. However, ketoprofen, oral or topical, can cause photosensitization through the formation of radical species following absorption of UVR [251,253]. When

exposed to UVR, ketoprofen can cause lipid peroxidation, DNA adduct formation, and a photoallergic reaction [251]. Nakajima, et. al. have demonstrated that quercetin strongly suppresses free radical generation by light exposed ketoprofen, suggesting that quercetin may be effective in protecting against photosensitization [252].

2.5.4 Summary

The yellow polyphenol quercetin is a sparingly water soluble plant derived flavanol with absorption bands in the UVC and UVA but no fluorescence chromophores. Quercetin is also susceptible to oxidation and up to 20 oxidation products have been observed. Of these twenty products, the most commonly observed and most abundant are protocatechuic acid (3,4-Dihydroxybenzoic Acid), phloroglucinol carboxylic acid (2,4,6-Trihydroxybenzoic Acid) and a phloroglucinol carboxylic acid - protocatechuic acid ester (quercetin depside) which are generated in a number of oxidation systems. Addition of radical scavengers such as GSH to quercetin oxidation systems has shown that GSH adds at the C6 and C8 positions, suggesting that the quercetin semi-quinone is oxidized to the *o*-quinone which isomerizes to the *p*-quinone methide which has electrophilic character at the C6 and C8 positions. However, due to the complex nature of quercetin oxidation, the complete mechanism is still not clear.

Limited work has also been done on the photochemical properties of quercetin. Although photobleaching in solution has been observed, this was in the presence of bubbled oxygen suggesting the possibility of oxidation by photo-induced singlet oxygen. Photobleaching of quercetin on deposited on cellulose has also been observed, resulting in the production of 3,4-dihydroxybenzoic acid being identified as a degradation product,

presumably being formed through the depside. However, to date the identity of the photodecomposition products of quercetin, if any, have not been reported.

Quercetin is generally found in plants as a glycoside and is high concentrations in food plants such as onion, lettuce, broccoli, cranberries and apple skins. As a result, most North Americans have a fairly high rate of quercetin intake, estimated at 25mg/day. Following ingestion quercetin glycosides pass through the small intestine to the colon where hydrolysis of the sugar occurs to give quercetin aglycone which is absorbed. In addition to the glycosides, many plants also contain small amounts of quercetin aglycone which is passively absorbed in the upper portion of the duodenum. Once in circulation, quercetin is highly protein bound.

Due to the five hydroxyl groups found on quercetin, the CYP enzymes do not act on it. However, quercetin has been found to modulate the activity of CYP 1A, 3A4, 3A3 and Cyp 2B1 in rats. Although not metabolized by the CYP enzymes, quercetin is subject to conjugation by phase II enzymes. The conjugation products of quercetin include glucutronidates, methylates, sulphonates, and multiple conjugates involving any combination at a variety of positions. Following conjugation, quercetin is usually excreted in the urine.

Since quercetin is readily oxidized, it is also an effective anti-oxidant and a large amount of research has been done on this property. A number of anti-oxidant measurement systems have been used to determine just how effective an anti-oxidant quercetin is and have resulted in a range of values. Trolox equivalent values of 4.7mM and 2.5mM have been reported as well as IC_{50} values ranging from 1.7µM to 200µM. Stoichiometric values for quercetin's anti-oxidant potential are less variable but still

range from 2.35 to 3.7 radicals per molecule. This anti-oxidant values are derived from a variety of analytical techniques in a variety of systems, but clearly no consensus exists on how effective an anti-oxidant quercetin is. In addition to the anti-oxidant capacity of quercetin, the structural components that make it an effective radical scavenger have been determined which have been shown to be the catechol group on the B-ring, the double bond between carbons 2 and 3, and the presence of a 3 hydroxyl and a 5 hydroxyl on the A ring along with the C-4 oxo-group.

Quercetin also has a number of biological effects in addition to its anti-oxidant properties. In rats, quercetin has been shown to decrease endogenous anti-oxidant enzyme levels while increasing levels of glutathione as well as inhibiting AP-1 and the JNK pathway. In addition, quercetin has been shown to cause up-regulation of melanin synthesis, suggesting a possible mechanism of UVR protection.

Quercetin, quercetin derivatives and their glucosides have been shown to be upregulated in a number of plant species in response to UVR exposure including *Brassica napu*s, *Petunia axillari,* and *Malus domestica.* Although the extract mechanism of action remains unclear, it is believed that this up-regulation results in a photoprotective effect. Quercetin administered by a number of routes has also been investigated for its photoprotective effects in mammals. Oral quercetin did not prevent onset of NMSC's but did prevent contact hypersensitivity in mice while topical and IP administered quercetin prevented oxidative stress in skin. In addition quercetin has also been shown to reduce expression of the markers of photoaging and prevent photosensitization by the NSAID ketoprofen.

In summary, the plant polyphenol quercetin has a number of physiochemical properties that result in it being a strong anti-oxidant as well as effecting enzyme levels and showing various photoprotective properties. These findings suggest that a topically applied formulation of quercetin may be effective in reducing the negative results of UVR exposure. The investigation of this possible means of sun protection is the focus of the research presented here.

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3. Hypothesis and Objectives

Due to the deleterious effects of UVA and UVB radiation and the uncertainties associated with currently available commercial sunscreens, investigations into a new, natural product based sunscreen may prove useful. We hypothesise that quercetin (3,3',4',5,7-Pentahydroxyflavone), when applied topically, provides protection from damage due to UVB and UVA radiation by UVB/UVA radiation absorption, antioxidant activity and interference with cellular signalling pathways.

In order to test this hypothesis, four major objectives for this research project were established.

3.1 Objective 1 – Determination of the antioxidant capacity of quercetin in a simulated membrane system

One of the major mechanisms by which UVR causes deleterious effects in the skin is through the generation of reactive oxygen species [49,50]. As such, clearly defining the anti-oxidant capacity of quercetin against both chemical oxidants and UVR is important for determining its ability to protect against UVR induced skin damage.

3.2 Objective 2 – Determination of the photo-stability of quercetin and identification of quercetins photodecomposition products

One of the concerns about some of the currently available sunscreens is the question of photostability. If the sunscreen breaks down on exposure to UVR the effectiveness is likely to decrease and the possibility of toxic product formation exists. As such it is important to access the photostability of quercetin and to identify any decomposition products.

3.3 Objective 3 – Determination of quercetin's ability to prevent photo-sensitization due to decomposition of the xenobiotic ketoprofen

In addition to directly causing harmful effects in the skin, UVR can also react with exogenous compounds such as pharmaceuticals to cause damage. One such pharmaceutical is the NSAID ketoprofen which has been shown to cause photosensitization [251,253]. As such, the ability of quercetin to prevent photo-sensitization using ketoprofen as a model needs to be determined.

3.4 Objective 4 – Determination of the effect of quercetin on biomarkers of photo-aging and photo-induced DNA damage

Two of the major endpoints of UVR induced damage are photoaging and damage to DNA. Assessment of quercetin's ability to prevent both DNA damage and photoaging are therefore necessary for determining it's suitability as a sunscreen.

4. Inhibition of UVA and UVB Radiation-Induced Lipid Oxidation by Quercetin

Brian M. Fahlman and Ed S. Krol

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Lab based experiments were performed by Brian Fahlman with supervisory consultation with Ed Krol.

Paper was written by Brian Fahlman and Ed Krol

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5. UVA and UVB radiation-induced oxidation products of quercetin

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6. UV Irradiation of Quercetin in the Presence of Ketoprofen, but not Oxybenzone, Leads to C-Ring Breakdown Products.

Brian M. Fahlman and Ed S. Krol

Prepared for submission to Journal of Photochemistry and Photobiology B : Biology

Lab based experiments were performed by Brian Fahlman with supervisory consultation with Ed Krol.

Paper was written by Brian Fahlman and Ed Krol

6.1 Abstract

The flavonol quercetin is currently being investigated for its potential as a topical skin photoprotectant. Quercetin can protect against the UV-mediated breakdown of ketoprofen, a non-steroidal anti-inflammatory drug associated with skin photosensitivity. We have previously examined the photostability of quercetin in methanol to UVA and UVB radiation and have determined that a small conversion (<10%) to C-ring breakdown products occurs over 24 hours. The major photoproducts from either UVA or UVB radiation were determined to be 2, 4, 6-trihydroxybenzaldehyde, 2-(3', 4'-dihydroxybenzoyloxy)-4, 6-dihydroxybenzoic acid and 3, 4-dihydroxyphenylethanol. This process was dramatically accelerated by the addition of a triplet sensitizer such as benzophenone. In the present work, we determined whether two structural analogs of benzophenone, ketoprofen and the sunscreen ingredient oxybenzone, could catalyze UV-mediated quercetin decomposition. We determined that ketoprofen catalyzes UV-mediated decomposition of quercetin in a similar manner to benzophenone. Conversely, oxybenzone does not catalyze UV-mediated quercetin breakdown and is stable to UV radiation. The ability of quercetin to spare ketoprofen from UV radiation-induced decomposition may indicate an important role for quercetin in addition to its potential as a topical photoprotectant.

Keywords
quercetin; ketoprofen; oxybenzone; photosensitization; sunscreen.

Classification codes

82.50.Hp, 82.30.Lp, 82.80.Bg

6.2. Introduction

The non-steroidal anti-inflammatory drug ketoprofen (Figure 6.1) is commonly used in the treatment of musculoskeletal and joint disorders, ankylosing spondylitits, osteoarthritis and rheumatoid arthritis which may be applied orally, rectally, intramuscularly or intravenously as the sodium salt [1]. In addition, ketoprofen can be used topically as a 2.5% topical gel for local pain relief [1]. Ketoprofen however, is known to cause photosensitization resulting in lipid peroxidation, DNA damage and photoallergic contact dermatitis [1]. Photosensitization in the skin is a process whereby light, most often ultraviolet (UV) radiation, is absorbed by an endogenous or exogenous chromophore, resulting in damage to the surrounding tissue [2]. This process often proceeds through the generation of reactive oxygen species (ROS) where the excited chromophore passes the excitation energy to oxygen by a type II photoreaction [3]. Alternatively, the excited state chromophore may pass the excitation energy to another biomolecule or undergo photoinduced changes itself in a type I photoreaction [3].

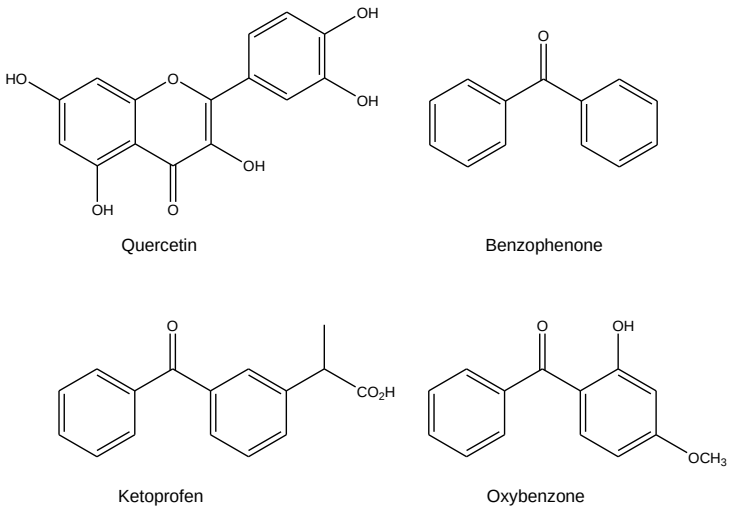

Figure 6.1 - Structures of quercetin, benzophenone, ketoprofen and oxybenzone.

The photosensitization process has a number of deleterious effects in the skin including lipid peroxidation [4,5], induction of inflammatory cytokines [6], alteration of cell signalling processes [7-10], photoaging [11], immunosuppression [11, 12], DNA damage [13] and necrosis [11]. In addition to ketoprofen, a number of pharmaceutical and natural health products can also act as photosensitizers. These include, but are not limited to, anti-inflammatory drugs such as naproxen, Central nervous system drugs such as proxibarbital, cardiovascular drugs such as digitoxin, and vitamins such as retinol [254]. Human skin also contains a number of endogenous photosensitizers which may contribute to photosensitization including bilirubin [10], urocanic acid, FMN, FAD [15], NADH, tryptophan, riboflavin [8], quinones [10], advanced glycation end-products formed on intra- and extra-cellular proteins [16, 17], unmodified extra-cellular proteins [18,19] and DNA [20].

Ketoprofen likely causes photosensitization through the generation of reactive oxygen species and other radicals. UV radiation exposure of ketoprofen has been shown to produce ROS including superoxide radical, hydroxyl radical and singlet oxygen [21]. Ketoprofen exposed to UV radiation also produces radical species which have not been identified, although a ketoprofen carboxylic acid radical which undergoes decarboxylation to form a 3-(benzophenyl) ethane radical has been proposed [21]. A variety of radical scavengers, notably the plant polyphenol quercetin, have been shown to prevent the photodecomposition of ketoprofen and production of ROS [22].

Quercetin (Figure 1) is currently under investigation for use as a topically applied sunscreen and has proven to be effective in preventing UV radiation-induced lipid peroxidation [23] as well as UV radiation-induced production of biomarkers of UVA

(matrix metalloprotease-1) and UVB (tumor necrosis factor-α) radiation *in vitro* (Fahlman and Krol, manuscript in preparation, see Chapter 6). Quercetin is relatively stable to UV radiation *in vitro* as UV irradiation of quercetin results in the slow formation of three photoproducts [24]. The UV radiation-mediated decomposition of quercetin is however greatly accelerated by addition of the triplet state sensitizer benzophenone (Figure 1). The aryl ketone portion of benzophenone is critical to its ability to act as a triplet sensitizer [25] and ketoprofen shares this structural feature (Figure 6.1). The previous study which identified that quercetin could spare UV radiation-induced ketoprofen decomposition did not indicate the fate of quercetin after the UV exposure [22], and we hypothesize that ketoprofen might act as a UV sensitizer for quercetin in a manner similar to benzophenone. In addition, the sunscreen ingredient oxybenzone (Figure 1) shares the same aryl ketone structural feature. There is one report suggesting oxybenzone is unstable to UV radiation [26], although other studies have found that oxybenzone appears to be stable to UV radiation [27, 28]. In our studies we saw no significant loss of benzophenone when used as a triplet sensitizer [24], so it is possible that oxybenzone stability to UV radiation may not exclude triplet sensitization.

There are two major objectives of this study, the first is to determine if the products derived from UV radiation of quercetin in the presence of benzophenone are also observed in a quercetin-ketoprofen irradiation. This would infer an excited state energy transfer protective mechanism for quercetin that may be a unique photoprotective property. The second objective is to determine whether oxybenzone can act as a UV sensitizer for quercetin decomposition and if quercetin spares any oxybenzone decomposition.

6.3 Materials and Methods

6.3.1 Materials

Quercetin, ketoprofen and oxybenzone were purchased from Sigma (St. Louis, MO). All solvents were HPLC grade. Water was purified using a Millipore Super Q water system with one carbon cartridge followed by two ion exchange cartridges (Bedford, MA).

2 FS20T12/UVB lamps (National Biological Corp., Beachwood, OH) filtered to remove UVC with an intensity of 1300 $\mu W \bullet cm^{-2}$ at 310 nm as measured with a UVP UVX-31 sensor or 2 F20T12/BL/HO UVA lamps (National Biological Corp., Beachwood, OH) filtered to remove UVC with an intensity of 740 $\mu W \bullet cm^{-2}$ at 365 nm as measured with a UVP UVX-36 sensor were used for irradiation.

6.3.2 High Performance Liquid Chromatography - Photodiode Array (HPLC-PDA)

HPLC-PDA analysis was carried out at room temperature on a Waters 600 system using a Waters 2996 photodiode array detector set at 365 nm. Aliquots of 50 μL were injected onto a 250 x 4.6 mm Allsphere ODS-2 column, 5 μm particle size (Alltech, Calgary AB). Data was processed using Empower software (Waters, Milford MA). Elution was carried out in gradient mode using two components: A = 0.1% formic acid in water, B = 0.1% formic acid in methanol. For quercetin and ketoprofen analysis the gradient was as follows: 0 to 10 min, linear gradient from 90% A to 40% A; 10 to 25 min, isocratic 40% A; 25 to 28 min, linear gradient from 40% A to 90% A; 28 to 30 min, isocratic 90% A. For oxybenzone analysis the mobile phase was the same and the gradient was 20 min, linear gradient from 90% A to 40% A; 20 to 65 min, isocratic 40% A; 65 to 68 min, linear gradient from 40% A to 90% A; 68 to 70 min, isocratic 90% A. Flow rate was 1.2 mL/min.

6.3.3 UV Irradiation

For photostability analysis of pure compounds 25mL of 50µM of quercetin, ketoprofen or 250µM oxybenzone in methanol were placed in open 30mL culture dishes and exposed to UVA (740 µW•cm^{-2} at 365 nm) or UVB (1300 µW•cm^{-2} at 310 nm) for 2 hours. Solvent volume was monitored every 15 min and methanol was added to maintain a volume of 25mL.

For photosensitization studies, 14 x 5mL samples of 50µM quercetin and 250µM benzophenone in methanol were placed in open 10mL culture dishes and exposed to UVA (740 µW•cm^{-2} at 365 nm) or UVB (1300 µW•cm^{-2} at 310 nm) for 2 hours. Samples were collected every 2.5 minutes for the first 15 minutes and then every 15 minutes until the end of the exposure time (120 minutes). To collect time points, one 10mL culture dish was removed from under the lamp, and the contents volume corrected to 5mL to compensate for loss due to evaporation. 150µL of the volume corrected sample was taken for analysis by HPLC-PDA.

6.3.4 Photoproduct Identification

Photoproducts were identified by retention time and UV spectra comparison to previously identified HPLC-PDA peaks [24]. (see Chapter 5)

6.3.5 Statistical Analysis

Statistical analysis was carried out using a Student's t-test performed with Microsoft Excel.

6.4. Results and Discussion

6.4.1 Photostability of Quercetin

UVA and UVB exposure of 50µM quercetin in methanol has previously been shown to result in less than 20% loss of starting material in 11 hours leading to low yields of

three photoproducts [24]. These photoproducts have been identified as 2,4,6-trihydroxybenzaldehyde, quercetin depside and hydroxytyrosol. The complete conversion of quercetin to the three photoproducts however can be accomplished in 1 h when the irradiation is carried out in the presence of 250 µM benzophenone, a triplet sensitizer [24] (see Chapter 4). Benzophenone itself is however stable under our conditions as we saw no decrease in benzophenone levels after all of the quercetin had undergone conversion to photoproducts (data not shown).

It is important to note that the total UV exposures used in these experiments are based on those from our previous study which are the doses required to attain complete conversion of quercetin to photoproducts [24]. While the UVA dose of 293 kJ/m^2 is environmentally relevant [29], the total UVB dose of 516 kJ/m^2 used is much greater than a normal environmental dose of UVB [30, 31].

6.4.2 Photostability of Ketoprofen

Our precise experimental setup differs from that of Nakajima [22], however UVA and UVB exposure of ketoprofen dissolved in methanol using our *in vitro* system resulted in complete loss of ketoprofen in two hours, which corresponds to the previously published results [21, 22] (Figure 6.2). As indicated earlier for quercetin, the UV doses used in these studies are required for complete photodecomposition of ketoprofen and at least in the case of the UVB dose, exceeds that expected for an environmentally relevant level of exposure [24].

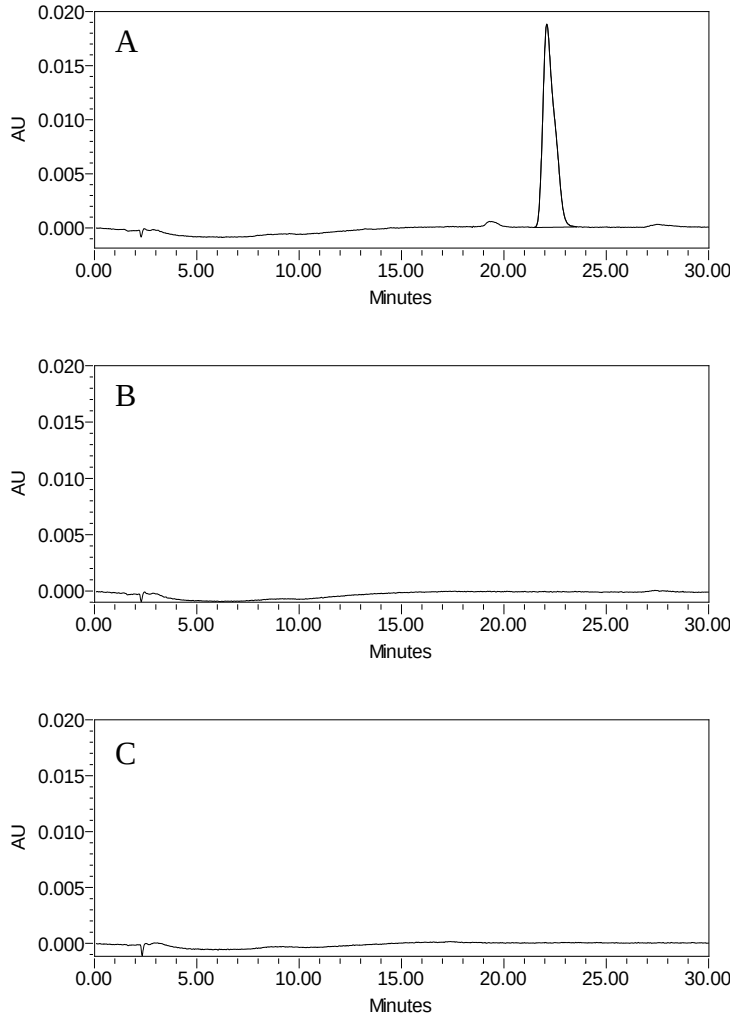

Figure 6.2 - Photostabilityof ketoprofen in methanol. 50µM ketoprofen in methanol was exposed to (a) no UVR, (b) UVA (740 µW•cm^{-2} at 365 nm) or (c) UVB (1300 µW•cm^{-2} at 310 nm) in uncovered 30mL culture dishes for 2 hours. Chromatograms were monitored at 302nm.

The original report on UV radiation-induced ketoprofen decomposition observed several decomposition products that eluted after the parent [22]. Under our HPLC conditions we were unable to observe any ketoprofen decomposition products. Our HPLC conditions were optimized for separation of the quercetin photoproducts and thus differed substantially from those used in the original report. The original ketoprofen study [22] utilized isocratic HPLC conditions which had a higher organic solvent content

than our gradient conditions, such that any ketoprofen decomposition products may have been obscured by the methanol wash conditions at the end of our gradient.

Exposure of 250μM ketoprofen to UVB (1300μW•cm^{-2} at 310 nm) for 1 h in the presence of 50μM quercetin resulted in no loss of ketoprofen, but partial loss of quercetin and the formation of the three quercetin photoproducts identified previously [24] (See Chapter 5) (Figure 6.3B). After 2 h of UVB exposure, all of the quercetin had undergone decomposition while only a minor loss of ketoprofen (<10%) was observed (Figure 6.3C). Similar results were seen for UVA (data not shown).

A time course study of the UV radiation-induced loss of quercetin and ketoprofen and the formation of quercetin photoproducts using both UVA and UVB radiation was performed (Figure 6.4). Figure 6.4 demonstrates that noticeable quercetin loss occurs within 2.5 min with concurrent formation of the three photoproducts for both UVA and UVB. Loss of ketoprofen however is minimal until all of the quercetin has been lost (Figure 6.4).

However, once all of the quercetin had been depleted, loss of ketoprofen occurred in both UVA and UVB exposed samples, with loss in the UVB sample occurring more rapidly (Figure 6.4). The results for UVA and UVB are similar to our previous results for UV radiation-induced quercetin decomposition in the presence of benzophenone, in which loss of quercetin occurs more rapidly under the UVB conditions [24]. Ketoprofen has been reported to form a triplet biradical upon UV irradiation [32], and this biradical may behave in a similar manner to the triplet sensitizer benzophenone. A triplet sensitization process would be in line with our and other observations for quercetin-mediated sparing of the UV radiation-induced decomposition of ketoprofen [22].

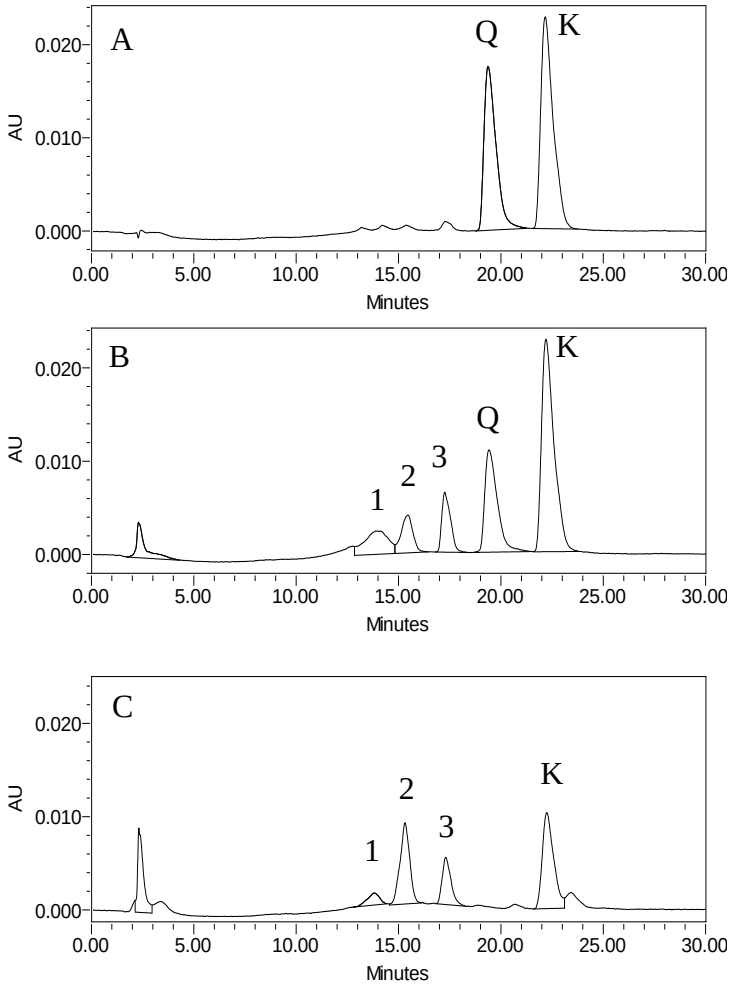

Figure 6.3 - Photostabilization of ketoprofen by quercetin in methanol. (a) 250µM ketoprofen in methanol in the presence of 50µM quercetin was exposed to UVB (1300µW•cm^{-2} at 310 nm) in uncovered 10mL culture dishes for (A) 0 hours (B) 1 hour or (C) 2 hours. Chromatograms were monitored at 302nm. K = Ketoprofen, Q = quercetin, 1 = 2,4,6-trihydroxybenzaldehyde, 2 = Quercetin depside, 3 = Hydroxytyrosol.

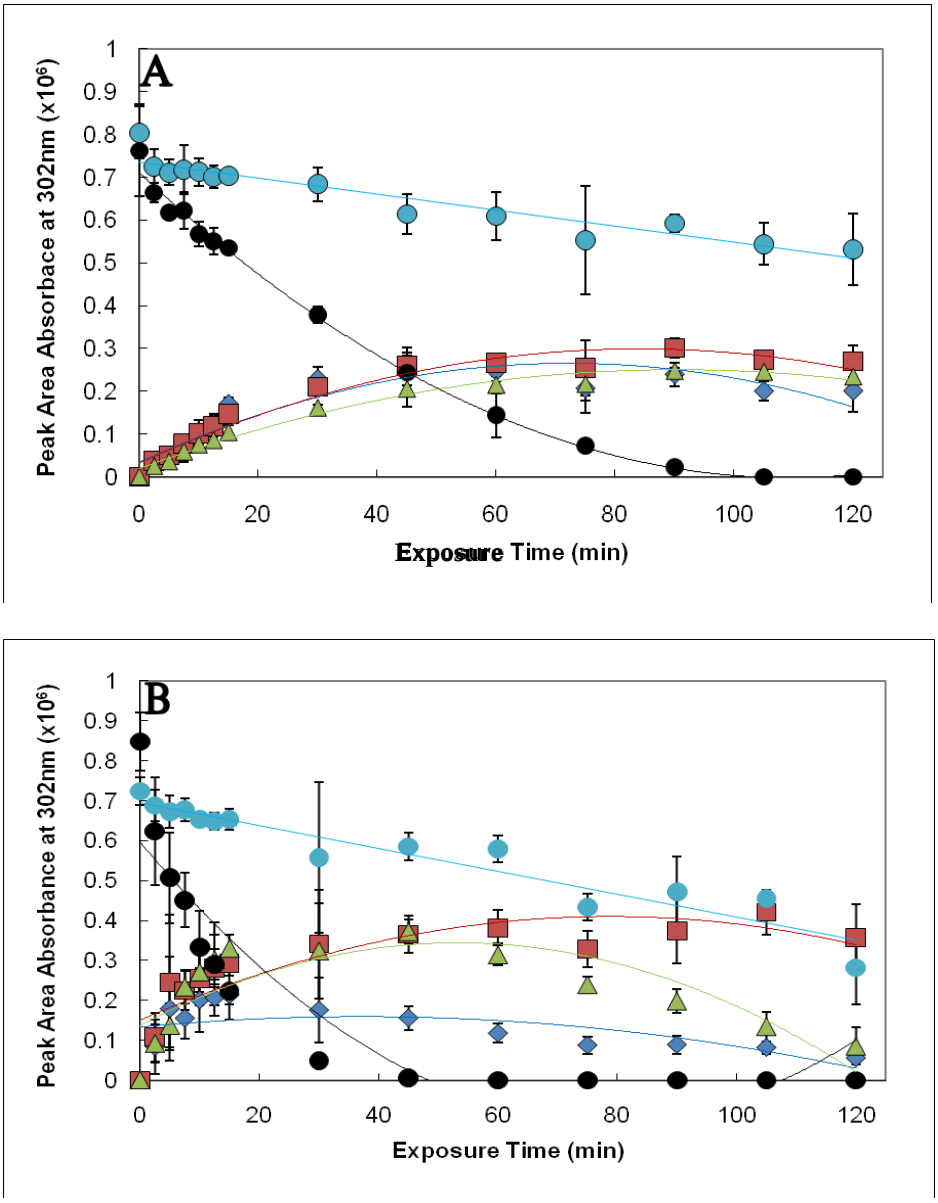

Figure 6.4 - Time course for UV radiation-induced decomposition of quercetin (50 µM in methanol) and ketoprofen and formation of quercetin-derived decomposition products, in the presence of ketoprofen (250 µM) (A) UVB radiation (1300 µW•cm^{-2}, 310 nm) for 2h, (B) UVA radiation (740 µW•cm^{-2}, 365 nm) for 2 h. Individual time points are the peak areas from HPLC chromatograms at 302 nm, of the volume corrected reaction mixture. Error bars represent standard deviation from the mean of three replicates. ♦ = Product 1, ■ = Product 2, ▲= Product 3, ●= Quercetin, ○ = Ketoprofen

6.4.3 Photostability of Oxybenzone

UVB exposure of 250µM oxybenzone in methanol for two hours in open culture dishes resulted in no loss of starting material (Figure 6.5A). Similar results were observed when UVA radiation was used in place of UVB (data not shown). When 50µM quercetin was exposed to UVB in the presence of 250µM oxybenzone, no increase in the rate of formation of quercetin photoproducts was observed compared to quercetin alone (Figure 6.5B). The photostability of oxybenzone in our system, combined with the absence of an increase in quercetin photoproducts as observed when quercetin was irradiated in the presence of ketoprofen or benzophenone, indicates that oxybenzone is not acting as a photosensitizer. Our results are consistent with previous reports suggesting that oxybenzone is stable to UV radiation [33, 34]. It has been suggested that this stability is due to an intramolecular hydrogen bond between the carbonyl and the 2-hydroxyl group of oxybenzone that leads to a fast, radiationless decay via tautomerization [35], thus avoiding a triplet state. This hypothesis is in agreement with our results as neither ketoprofen nor benzophenone possess the required 2-hydroxyl group (Figure 6.1).

In conclusion we have determined that ketoprofen can increase the UV radiation-mediated decomposition of quercetin producing the same photoproducts as observed when benzophenone is used, and we suggest that ketoprofen acts as a triplet sensitizer in a similar manner as benzophenone. Our results also support previous observations that quercetin can spare UV radiation-induced ketoprofen breakdown [1, 22]. As a result of this specific mechanistic interaction between ketoprofen and quercetin, these results suggest a potential photoprotective property of quercetin for the control of

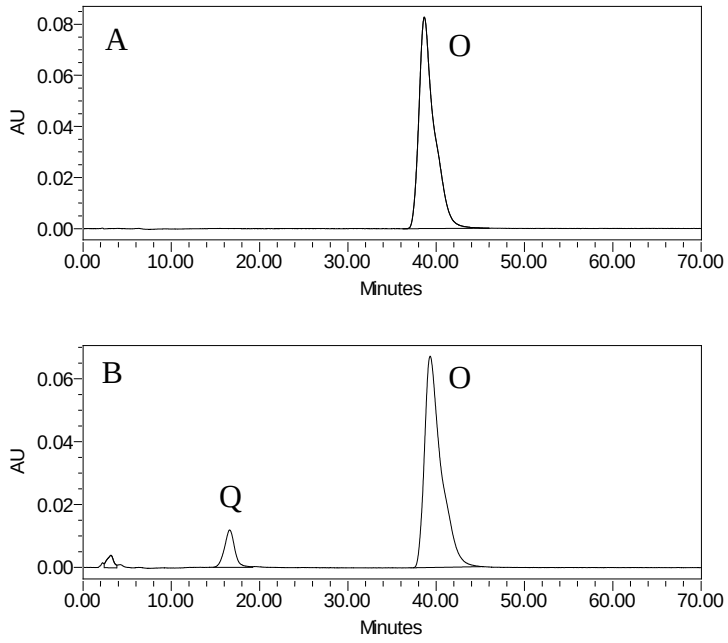

Figure 6.5 - Photostability of oxybenzone and photosensitization by oxybenzone in methanol. 250µM oxybenzone in methanol was exposed to UVB (1300µW•cm^{-2} at 310 nm) in uncovered 30mL culture dishes in the (A) presence or (B) the absence of 50µM quercetin for 2 hours. Chromatograms were monitored at 302nm. O = oxybenzone, Q = quercetin.

ketoprofen photosensitivity. Whether quercetin can minimize UV radiation-induced ketoprofen skin sensitivity *in vivo* remains to be determined.

We also confirmed that oxybenzone is stable to UV radiation and does not act as a triplet sensitizer with the result that oxybenzone does not catalyze the UV radiation-mediated decomposition of quercetin. It is interesting however to note a study in which oxybenzone was applied topically to artificial skin equivalents and the production of UV radiation-induced ROS was determined [34]. That study showed that oxybenzone was initially effective at decreasing UV radiation-induced ROS formation, however upon absorption into the epidermal layer, oxybenzone resulted in an increase in ROS formation. The authors speculate that this may be due to triplet sensitization by

oxybenzone as the result of the highly lipophilic environment in the cell. Whether quercetin could spare this oxybenzone-mediated ROS formation is unknown, but is an interesting hypothesis that warrants further investigation.

6.5. Abbreviations

Reactive oxygen species: ROS; High Performance Liquid Chromatography - Photodiode Array: HPLC-PDA; Ultraviolet: UV.

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7. The effects of quercetin of secretion of the biomarkers MMP-1 and TNF-α in artificial skin mimics

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Prepared for submission to Photodermatology, Photoimmunology and Photomedicine

Lab based experiments were performed by Brian Fahlman with supervisory consultation with Ed Krol.

Paper was written by Brian Fahlman and Ed Krol

7.1 Abstract

Exposure of skin to ultraviolet light has been shown to have a number of deleterious effects including photo-aging, photo-immunosuppression and photo-induced DNA damage which can lead to the development of skin cancer. The plant polyphenol quercetin is currently under investigation for potential use as a topically applied sunscreen affording protection from these negative effects. In this paper we present an assessment of quercetin to prevent induction of the photo-damage biomarkers matrix metalloprotease 1 (MMP-1) and tumor necrosis factor α (TNF-α) in HaCaT cell cultures and EpiDerm skin mimics.

In HaCaT cell cultures dosed with quercetin in media 24 hrs prior to UVA or UVB exposure, a concentration of 100μM was found to significantly decrease secretion of MMP-1 in response to both UVA and UVB while no change was seen in TNF-α secretion at any of the quercetin concentrations tested. In EpiDerm samples treated topically with quercetin immediately prior to UVA or UVB exposure, a concentration of 100μM quercetin significantly decreased UVA induced MMP-1 secretion, but 200μM quercetin was required to significantly reduce UVB induced MMP-1 secretion. TNF-α sectretion in EpiDerm samples was significantly reduced at 100μM quercetin for both UVA and UVB radiation. In addition, topically applied quercetin was found to be photo-stable over the duration of the experiment.

7.2. Introduction

Ultraviolet radiation (UVR), defined as electromagnetic radiation in the range of 100nm to 400nm, accounts for roughly 10% of the solar radiation that reaches the earth [1]. Of this radiation, the ultraviolet C (UVC) portion (<280nm) and 90% to 99% of the ultraviolet B (UVB) (280nm-315nm) is filtered by atmospheric ozone, leaving ultraviolet A (UVA) (315nm-400nm) as the major component of ultraviolet radiation to reach the earth's surface [1].

The ultraviolet radiation that reaches the earth's surface has a number of effects on mammalian systems. Positive effects of UVR include regulation of circadian rhythm and of the conversion of 7-dehydrocholesterol to pre-vitamin D_3, a precursor of vitamin D, which has a wide range of beneficial effects as reviewed by Goltzman [2] and Dixon et. al. [3]. Exposure to ultraviolet radiation also has a number of negative biological effects including lipid peroxidation leading to cell dysfunction or necrosis [4,5] destruction of skin collagen leading to photoaging [6], immunosuppression [7], and DNA damage leading to skin cancer, through either oxidative [8] or direct photochemical processes [9,10].

UVR induced photoaging is primarily associated with the UVA wavelengths and is characterized by increased wrinkle formation relative to unexposed skin, a loss of skin recoil capacity and an increase in skin fragility [11-13]. Histologically, the most prominent feature of photoaged skin is solar elastosis, characterized by degradation of collagen and accumulation of abnormal elastin [13,14]. UVA, and to a lesser extent UVB radiation, causes photoaging through induction of matrix metalloprotease-1 (MMP-1) which is responsible for the breakdown of collagen [11,15-19].

The molecular pathway leading from UVR exposure to induction of the matrix-metalloproteases and photoaging is under investigation although it remains incomplete. It is well known that both UVA and UVB radiation can induce the formation of reactive oxygen species (ROS) in the skin [4,20] and there is good evidence that it is these ROS that initiate the photoaging process. First, the level of H_2O_2, an inducer of MMP-1 [21], has been found to be elevated in photoaged skin relative to naturally aged skin, perhaps accounting for the differences in MMP activities [15]. Secondly, it has been shown that dark reaction produced $^1O_2$, which can be converted to form H_2O_2, induces MMP-1 mRNA in the same dose dependant manner as UVA in cultured fibroblasts [21]. In addition, exposure of fibroblasts to UVA resulting in MMP-1 upregulation has been shown to be enhanced by the addition of D_2O, which increases the lifetime of $^1O_2$ [22], and that addition of sodium azide, a quencher of $^1O_2$, dramatically decreases the expression of MMP-1, implicating $^1O_2$ in the process [23]. Taken together, these facts may show that the formation of ROS is the first step in photoaging**.**

The enzymatic pathway connecting ROS to photoaging has been filled in by a number of groups and appears to mediated through several interleukins. It has been determined by Wlaschek, et. al. that fibroblasts exposed to UVA show an upregulation in IL-6 mRNA and protein and that inhibition of the translation of IL-6 prevents MMP-1 upregulation, showing that IL-6 is necessary for increases in MMP-1 activity [12]. This same group later showed that following UVA exposure, there is an initial increase in the release of existing IL-1 which triggers the *de novo* synthesis of IL-6 which leads to MMP-1 induction and IL-1 which perpetuates the UV response [24].

Exposure to ultraviolet radiation, particularly UVB, has also been associated with immunosuppression, both local and systemic [6,7]. This immunosuppression is believed to reduce the risk of harmful, excessive inflammation in the skin following UVR exposure [6] and has been found to result from both acute [7] and chronic [25] exposure to UVR. UVB induced immunosuppression occurs via down regulation of T-cell mediated immunity through the induction of immunomodulating cytokines [6,26]. UVB radiation has been shown to induce IL-10 [27,28], IL-4[28], IL-6 [29] and TNF-α [28].

The various interleukins induced by UVB radiation appear to have different functions in the immunosuppressive effect. When IL-6 knockout mice are exposed to UVB, the loss of contact hypersensitivity seen in wild type mice as well as the increase in IL-6 and IL-10 levels in the blood were absent [29]. However, when the knockout mice were injected with IL-6, the IL-10 levels increased and immunosuppression was observed, supporting a role for UVB mediated induction of IL-6 which in turn induces IL-10 leading to immunosuppression [29]. This same study indicated that the cells responsible for IL-6 production appeared to be Langerhan's cells [29].

The initial skin chromophore involved in immunosuppression has been shown to be DNA, and UVR absorption by DNA resulting in the formation of thymine dimers is also the initiating step in photo-carcinogenesis [9,30]. UVB radiation is directly absorbed by pyrimidine residues, especially thymine. These excited residues then react with adjacent pyrimidine residues forming pyrimidine dimers [31,32]. These dimers can be formed between two adjacent thymines (T<>), two adjacent cytosines (C<>C) or between adjacent thymine-cytosine pairs (T<>C). The pyrimidine dimers can take a number of forms including 6-4 dimers and their Dewer isomers or most commonly cyclo-pyrimidine

dimers (CPD's) which are formed by [2+2] cycloaddition between the C5-C6 double bonds of adjacent pyrimidines [31,33]. The relative frequency of occurence of these dimers in sun exposed skin is T<>T > T(6-4)C > T<>C > T(6-4)T [31].

Yarosh, et. al. [34] demonstrated that in HaCaT cells UVB radiation induces the immunosuppressive cytokine TNF-α, and that this same cytokine was induced when DNA was damaged using *HindIII*. The same group also showed that increased repair of CPDs by addition of T4 endonuclease V, which is specific for UVR induced DNA damage, resulted in a decrease in immunosuppression [34]. Similar results have shown that in *in vivo* human experiments, T4 endonuclease V given after SSR exposure prevented immunosuppression [35].

If photo-induced dimers are not repaired prior to replication, they can result in C to T and CC to TT transversions. If these mutations occur in a protein coding region, particularly in the coding region for a regulatory protein such as p53, they can lead to the formation of carcinomas [6,36]. These photoinduced skin carcinomas fall into two broad categories, melanomas and non-melanomic skin carcinomas (NMSC) [8]. Melanomas, which are associated with a small number of intense UVR exposures (sunburns) occur when the UVR induced DNA damage takes place in melanocytes and account for roughly 4% of skin carcinomas [37]. Although relatively rare, melanomas are aggressive and likely to metastasise, resulting in a high mortality rate [37]. The majority of skin carcinomas are NMSC which are sub-divided into squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) [36]. Both types of NMSC are associated with cumulative, long-term UVR exposure and are caused by mutations in the keratinocytes [6]. NMSC

are generally less likely to metastasise than melanomas and generally result in disfigurement rather than mortality [37,38].

Due to the negative effects associated with overexposure to solar UVR, a large number of topically applied sunscreens are available on the market and the development of new sunscreens is an active area of research. One of the approaches being used in the development of new sunscreen products is the incorporation of natural products produced by plants for the purpose of UVR protection [39-41].

One phytochemical which is believed to have photoprotective properties in plants is the flavanoid quercetin (3, 3', 4', 5, 7-pentahydroxyflavone, Figure 7.1)). Quercetin has absorption bands in the UV range [42] and both the parent as well as various quercetin glycosides have been shown to be upregulated in response to UVR exposure in a number of plant species including Brassica napus [43], *Petunia axillaris* [44], *Malus domestica* [45]. Use of quercetin as a photoprotectant has proven effective in both human fibroblasts which showed a decrease in photoinduced MMP-1 production following quercetin treatment [46], and in mice where dietary intake of quercetin by mice has been shown to decrease UVR induced immunosuppression [47,48].

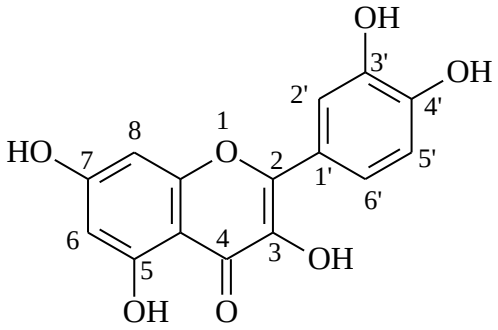

Figure 7.1 - Structure of quercetin (3, 3', 4', 5, 7-pentahydroxyflavone)

In addition to UVR absorbance, quercetin is also a strong antioxidant with each molecule capable of scavenging four radical species [49]. The potential for contribution from both an antioxidant and a UVR absorptive pathway has left the mechanism of quercetin photoprotection in mammalian systems unclear.

We hypothesize that quercetin can reduce UVR induced oxidative damage, including photoaging, and direct DNA damage in skin *in vitro*. To study this question we used HaCaT cell cultures and artificial skin mimics and determined the ability of quercetin to prevent UVR induced damage using MMP-1 and TNF-α as markers of UVR induced damage. Our results showed that quercetin added to cell culture media or applied topically to artificial skin mimics is effective in preventing the production of MMP-1, a marker for photoaging, and TNF-α, a marker for immunosuppression and a secondary marker for UVR induced DNA damage, specifically CPD formation. These results, together with our previous findings showing good photostability [50] and effective protection from photoinduced lipid peroxidation [49] indicate that quercetin may prove an effective component of a topically applied sunscreen. In this study, the endpoints studied were primarily photoaging and immunosuppression/direct DNA damage.

7.3. Methods and Materials

7.3.1 Chemicals

Quercetin was purchased from Sigma (St. Louis, MO). All solvents were HPLC grade. Water was purified using a Millipore Super Q water system with one carbon cartridge followed by two ion exchange cartridges (Bedford, MA).

2 FS20T12/UVB lamps (National Biological Corp., Beachwood, OH) filtered to remove UVC with an intensity of 1300 $\mu W \cdot cm^{-2}$ at 310 nm as measured with a UVP

UVX-31 sensor or 2 F20T12/BL/HO UVA lamps (National Biological Corp., Beachwood, OH) filtered to remove UVC with an intensity of 740 $\mu W \bullet cm^{-2}$ at 365 nm as measured with a UVP UVX-36 sensor were used for irradiation.

HaCaT cells were obtained from Cell Lines Service (Eppelheim, Germany) and cultured with Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) purchased from Invitrogen (Burlington, ON). EpiDerm™ skin mimics and media were purchased from MatTek Corporation, Ashland, MA.

Quantikine® Human pro-MMP-1 ELISA kits and Quantikine® Human TNF-α/TNFSF1A ELISA kits were purchased from Cedarlane Laboratories Ltd., Burlington, ON and read using a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski, VT).

7.3.2 Cell Cultures

7.3.2.1 HaCaT Cells

HaCaT cell cultures were grown as per the supplier's instructions. Briefly, $4x10^5$ cells were suspended in 15 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS), plated onto T-75 culture flasks and incubated at 37ºC with 5% CO_2. When cells reached 90% confluency they were treated with 0.05% EDTA / 0.1% trypsin in phosphate buffered saline (PBS) to detach and seeded onto 6 well plates at a density of $5x10^3$ cells/cm^2. The six well plates were incubated in DMEM containing 10% FBS at 37ºC with 5% CO_2 until 90% confluency was reached prior to treatment.

7.2.2.2 EpiDerm Skin Mimics

EpiDerm artificial skin mimics were purchased from MatTek (Ashland, MA) and used as per the supplier's instructions. Briefly, upon receipt of the samples, individual skin

mimics were placed in each well of a 6-well culture plate along with 1mL of maintenance media and incubated at 37°C with 5% CO_2 overnight. Following incubation, the maintenance media was replaced with assay media for UVR exposure.

7.3.3 Quercetin Treatment

7.3.3.1 HaCaT Cell Cultures

For dosing with quercetin, each well containing HaCaT cells at 90% confluency was washed with PBS, and 2mL DMEM with 10% FBS was added. To the media was added 0.5µL, 1.0µL, 2.0µL or 4.0µL of 65mM quercetin in DMSO to obtain concentrations of 25µM, 50µM, 100µM and 200µM respectively. Quercetin control contained 4µL DMSO and vehicle control had neither DMSO nor quercetin added to the media. Following dosing, cells were returned to the incubator at 37°C with 5% CO_2 for 24 hours. All doses were done in triplicate.

7.3.3.2 EpiDerm Skin Mimics

EpiDerm skin mimics were treated topically with quercetin in acetone immediately prior to UVR exposure. The amount of quercetin used was calculated to be the same mass of quercetin delivered on to the skin per cm^2 as was delivered to the HaCaT cells of concentrations of 100µM and 200µM. Skin mimics were treated with 50µL and 100µL of 26µM quercetin in acetone to achieve doses equivalent to 100µM and 200µM. Following application of the quercetin in acetone, samples were left for 10min to allow acetone to evaporate leaving a thin layer of quercetin on the skin prior to UVR exposure. All doses were done in triplicate.

7.3.4 UVR Exposure

7.3.4.1 HaCaT Cell Cultures

For UVR exposure cells were removed from the incubator after 24 hours and the media removed and replaced with PBS. Samples were then placed under lamps without covers. For UVB exposure, cells were placed 20cm from 2 FS20T12/UVB lamps (National Biological Corp., Beachwood, OH) which resulted in an intensity of 500μW/cm^2 at 310nm and the cells were exposed for 30 min for a total dose of 9000J/m^2. For UVA exposure, cells were placed 4cm from 2 F20T12/BL/HO UVA lamps (National Biological Corp., Beachwood, OH) which resulted in an intensity of 700μW/cm^2 at 365nm and the cells were exposed for 240 min for a total dose of 100kJ/m^2. For dark samples, plates were wrapped in aluminum foil and placed under 2 F20T12/BL/HO UVA lamps (National Biological Corp., Beachwood, OH) for 240 min to account for heating effects. Output of all lamps was filtered to remove UVC radiation. Following exposure, PBS was removed and replaced with 2mL fresh DMEM with 10% FBS and samples were returned to the incubator at 37°C with 5% CO_2. Cell cultures were removed from the incubator 24 hours after exposure and media collected and placed in a -80°C freezer for later analysis.

7.3.4.2 EpiDerm Skin Mimics

For UVR exposure skin mimics once the acetone vehicle had evaporated samples were placed under lamps without covers. For UVB exposure, cells were placed 20cm from 2 FS20T12/UVB lamps (National Biological Corp., Beachwood, OH) which resulted in an intensity of 500μW/cm^2 at 310nm and the samples were exposed for 30 min for a total dose of 9000J/m^2. For UVA exposure, cells were placed 4cm from 2 F20T12/BL/HO UVA lamps (National Biological Corp., Beachwood, OH) which

resulted in an intensity of 700μW/cm^2 at 365nm and the samples were exposed for 240 min for a total dose of 100kJ/m^2. For dark samples, plates were wrapped in aluminum foil and placed under 2 F20T12/BL/HO UVA lamps (National Biological Corp., Beachwood, OH) for 240 min to account for heating effects. Output of all lamps was filtered to remove UVC radiation. Following exposure samples were returned to the incubator at 37°C with 5% CO_2. Cell cultures were removed from the incubator 24 hours after exposure and media collected and placed in a -80°C freezer for later analysis.

7.*3.5 ELISA Analysis*

7.3.5.1 pro-MMP-1

Extracellular pro-MMP-1 produced in response to UVR in the presence or absence of quercetin was measured using a Quantikine® Human pro-MMP-1 ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's directions. Briefly, 100μL of culture media and 100μL of diluent solution were added to each well of a 96-well plate and shaken at room temperature for 2 hours. The samples were aspirated, the plate was washed four times and 200μL of conjugate was added to each well and the plate shaken at room temperature for 2 hours. Conjugate solution was aspirated, the plate was washed four times and 200μL substrate solution was added to each well followed by a 20 min incubation protected from light. 50μL of stop solution was then added to each well and the absorbance at 450nm and 570nm was recorded using a BioTek Instruments (Winooski, VT) plate reader. An eight point calibration curve was prepared using standards provided by the supplier and the pro-MMP-1 concentration of each well was determined by interpolation.

7.3.5.2 TNF-α

Extracellular TNF-α produced in response to UVR in the presence or absence of quercetin was measured using a Quantikine® Human TNF-α/TNFSF1A ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's directions. Briefly, 50µL of culture media and 200µL of diluent solution were added to each well of a 96-well plate and incubated at room temperature for 2 hours. The samples were aspirated, the plate was washed four times and 200µL of conjugate was added to each well and the plate incubated at room temperature for 1 hour. Conjugate solution was aspirated, the plate was washed four times and 200µL substrate solution was added to each well followed by a 20 min incubation protected from light. 50µL of stop solution was then added to each well and the absorbance at 450nm and 570nm was recorded using a BioTek Instruments (Winooski, VT) plate reader. An eight point calibration curve was prepared using standard provided by the supplier and the TNF-α concentration of each well was determined by interpolation.

7.3.6 Quercetin Stability

The stability of quercetin which was topically applied to the EpiDerm tissues was also assessed. Following UVA or UVB exposure 100µL of acetone was placed on the EpiDerm samples and allowed to re-dissolve the UVR exposed quercetin and photoporducts. The acetone was then collected and analyzed by HPLC-PDA as described previously [50] (Chapter 5).

7.3.7 Statistics

Statistical analyses were performed using GraphPad Prism 5.0 (GrpahPad Software Inc., La Jolla, CA). Data was analyzed by one-way ANOVA with a Bonferroni's Multiple Comparison Test. The level of significance was set at $P<0.05$.

7.4 Results

The ability of quercetin to prevent UVA and UVB induced production and/or release of MMP-1 and TNF-α was initially assessed in cultured keratinocyte mono-layers. In these experiments the keratinocyte cultures were dosed with appropriate amounts of quercetin in the cell media 24 hours prior to exposure to UVA or UVB and the media was removed prior to exposure. As a result, any photoprotective activities seen in this set of experiments would be due to quercetin that had been taken up by the cells during the dosing period. As such, the quercetin could be acting in an antioxidant capacity [49,51], a photoabsorptive capacity [43,47,48], an enzyme modulating capacity [46] or any combination of these mechanisms.

As can be seen from Figure 7.2, pre-treatment of HaCaT cells with 100µM and 200µM quercetin in media 24 hours prior to UVR exposure resulted in a significant decrease in the amount of MMP-1 released by the cells relative to both vehicle control (DMSO) and untreated cells. This significant decrease in MMP-1 release is seen in both UVA and UVB exposed cells, though the decrease is more dramatic in the UVA exposed cells. Pre-treatment of the HaCaT cells with 50µM quercetin 24 hours prior to UVR exposure shows a trend towards a decrease in MMP-1 release in both UVA and UVB exposed cells, but this decrease did not reach the level of significance due to high variability in the vehicle control and untreated samples.

The effects of pre-treatment with quercetin on the production of TNF-α by HaCaT cells is presented in Figure 7.3. In contrast to the results seen for MMP-1, pre-treatment of HaCaT cells did not result in a significant decrease in TNF-α production, even when doses up to 200μM quercetin were used.

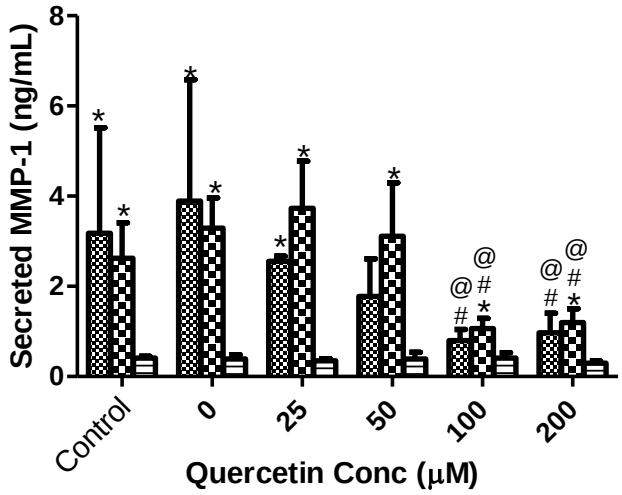

Figure 7.2 – Quercetin prevention of MMP-1 production by HaCaT cell cultures exposed to either 100kJ/m^2 UVA, 900J/m^2 UVB (in open culture dishes, UVC removed by filtration) or no UVR (dark) by ELISA. Cell cultures were treated with quercetin in DMSO (<2% by volume) added to media 24 hours prior to exposure. For UVR exposure, media was replaced with phosphate buffered saline, following treatment saline was replaced with DMEM and cells were returned to the incubator. Media was collected for pro-MMP-1 analysis 24 hours later and stored at -80ºC until analysis. = UVA, = UVB Treatment, = Dark Control. * = significantly different from dark control (P<0.05), # = significantly different from control (P<0.05), @ = significant different from 0μM (vehicle control) (P<0.05).

Following the preliminary study using cultured HaCaT cells, the effectiveness of quercetin as a photo-protectant was assessed using an EpiDerm^{TM} artificial skin mimic. In this case quercetin was applied topically to the stratum corneum of the skin mimics immediately prior to UVR exposure. In contrast to the HaCaT cell study where quercetin

was able to fully incorporate into the cell allowing for possible effects on cell signalling and intracellular antioxidant activity, topical application relies primarily on photo-absorptive processes and intercellular and cellular membrane antioxidant activity to provide photoprotection.

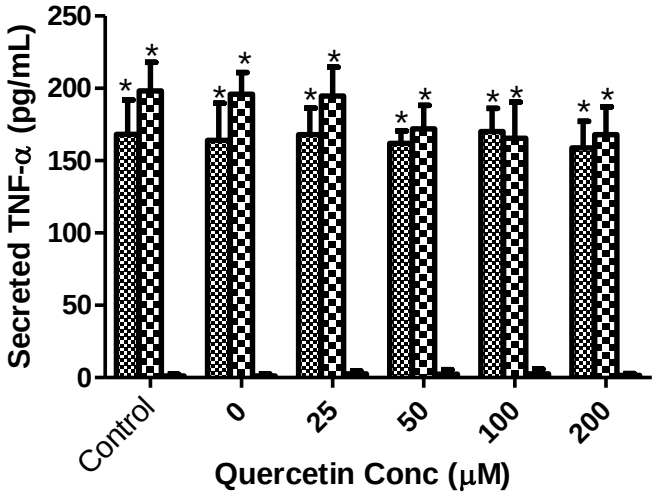

Figure 7.3 – Quercetin prevention of TNF-α production by HaCaT cell cultures exposed to either 100kJ/m^2 UVA, 900J/m^2 UVB (in open culture dishes, UVC removed by filtration) or no UVR (dark) by ELISA. Cell cultures were treated with quercetin in DMSO (<2% by volume) added to media 24 hours prior to exposure. For UVR exposure, media was replaced with phosphate buffered saline, following treatment saline was replaced with DMEM and cells were returned to the incubator. Media was collected for TNF-α analysis 24 hours later and stored at -80ºC until analysis. = UVA, = UVB Treatment, = Dark Control. * = significantly different from dark control (P<0.05)

6.3.2. UVR exposed EpiDerm™

The effect of topical quercetin treatment of EpiDerm™ on MMP-1 excretion is presented in Figure 7.4. As can be seen in this graph, both UVA and UVB exposed samples treated with the equivalent of either 100μM or 200μM quercetin resulted in a significant decrease in MMP-1 production compared to control. In fact, for both UVA

and UVB exposed EpiDerm™ treated with 200µM quercetin, extracellular production of MMP-1 decreased to the level seen in non-UVR exposed skin mimics (dark control).

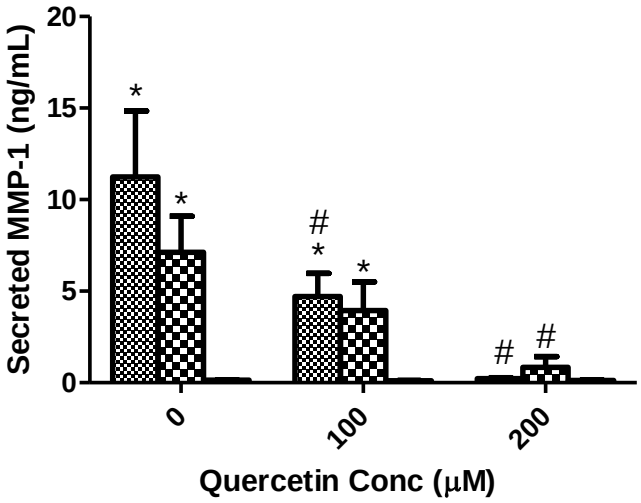

Figure 7.4 – Quercetin prevention of MMP-1 production by EpiDerm^{TM} skin mimics exposed to either 100kJ/m^2 UVA, 900J/m^2 UVB (in open culture dishes, UVC removed by filtration) or no UVR (dark) by ELISA. Cell cultures were treated with quercetin in acetone, which was allowed to evaporate, immediately prior to UVR exposure leaving a thin film of quercetin. Following treatment skin mimics were returned to the incubator. Media was collected for MMP-1 analysis 24 hours later and stored at -80ºC until analysis. = UVA, = UVB Treatment, = Dark Control. * = significantly different from dark control (P<0.05), # = significantly different from control (P<0.05).

Figure 7.5 shows the effects of topical quercetin treatment of the EpiDerm™ skin mimics on the production of TNF-α. For UVR treated EpiDerm™, TNF-α production shows a pattern similar to that seen for MMP-1 with significant decreases in both UVA and UVB-induced TNF-α production at 100µM and a complete loss of TNF-α production to dark control levels in those skin mimics treated with 200µM quercetin. In fact, for the UVA exposed samples, reduction to dark control levels of TNF-α was seen at 100µM

quercetin treatment. This is in contrast to the HaCaT cell experiments where quercetin had no effect on TNF-α production.

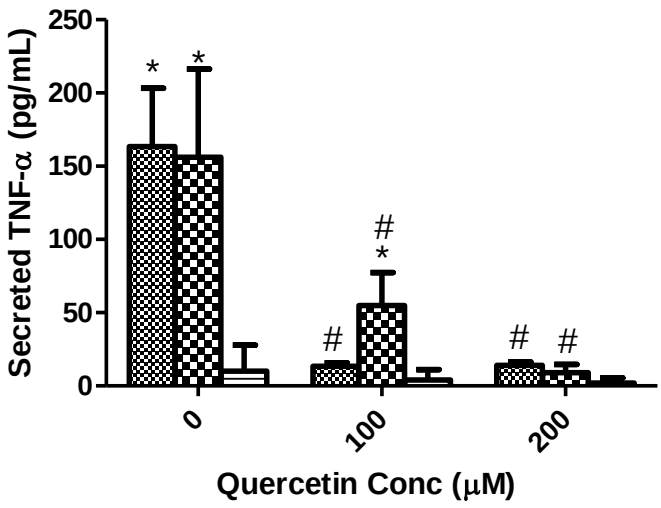

Figure 7.5 – Quercetin prevention of TNF-α production by EpiDerm^{TM} skin mimics exposed to either 100kJ/m^2 UVA, 900J/m^2 UVB (in open culture dishes, UVC removed by filtration) or no UVR (dark) by ELISA. Cell cultures were treated with quercetin in acetone, which was allowed to evaporate, immediately prior to UVR exposure leaving a thin film of quercetin. Following treatment skin mimics were returned to the incubator. Media was collected for TNF-α analysis 24 hours later and stored at -80ºC until analysis. = UVA, = UVB Treatment, = Dark Control. * = significantly different from dark control (P<0.05), # = significantly different from control (P<0.05).

Assessment of the photodegradation of quercetin applied topically to EpiDerm samples show that little loss observed as shown in Figure 7.6. As can be seen, the major component of the recovered photoprotectant is the parent compound quercetin with only trace amounts of the decomposition product quercetin depside [50] present. This stability of quercetin is in contrast to the decomposition of quercetin seen in DMEM [52], suggesting that the decomposition of quercetin observed was not due to UVR alone.

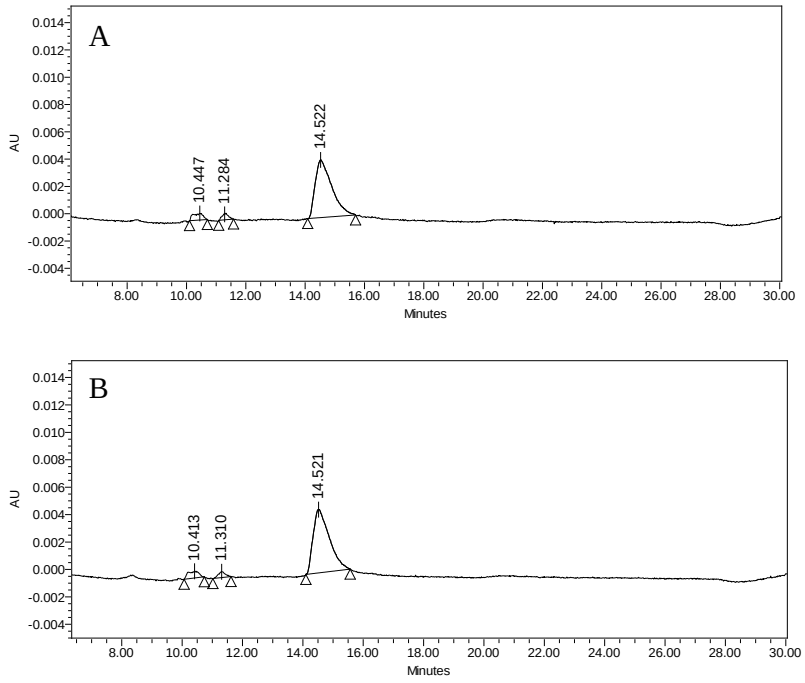

Figure 7.6 – HPLC analysis of quercetin exposed to UVA or UVB radiation on EpiDerm (A) UVA exposed quercetin (B) UVB exposed quercetin.

7.5. Discussion

A number of studies have suggested that flavonoids such as quercetin may have photoprotective properties. In an effort to develop a reliable model system to test topical photoprotectants, we decided to investigate the use of an artificial skin mimic as our model system. Two biomarkers of UVR induced damage were employed, MMP-1 secretion which is a marker of ROS mediated damage and photoaging effects and TNF-α, a marker of photo-immunosuppression and direct photodamage to DNA. We used this system to determine whether quercetin could provide some level of protection to skin from UVR. The study presented here shows that in both HaCaT cell culture systems and artificial skin mimics quercetin can reduce UVR induced photodamaging effects. Pilot studies carried out in cell culture systems determined that pre-treatment with quercetin 24

hours prior to UVA or UVB exposure resulted in decreased secretion of MMP-1, but had no effect on secretion of TNF-α. In contrast to this, topical treatment of artificial skin mimics with quercetin resulted in significant decreases in both MMP-1 and TNF-α excretion.

Two different routes of administration of quercetin were used in this study. The cell culture systems were used in an effort to establish appropriate concentration ranges of quercetin to be used in the human skin mimic (EpiDerm^{TM}) studies. In the case of the HaCaT cell cultures, quercetin was dosed into the cell media 24 hours prior to UVA and UVB exposure. This dosing protocol allows for absorption of quercetin into the cell prior to exposure to UVA or UVB radiation. A recent study determined that quercetin could induce apoptosis in UVB treated HaCaT cells, however the study also observed that quercetin was unstable in DMEM and decomposed to unknown products, although the extent of uptake of intact quercetin or decomposition products into the HaCaT cells is unknown [52]. Based on the known decomposition of quercetin [53] and our own investigations [50], we predict that the major decomposition product in DMEM is likely the quercetin depside. Interestingly, stabilization of quercetin in DMEM with ascorbic acid reduced the apoptotic effect [52]. The authors suggest that the apoptotic effect of quercetin may be affected by a combination of the parent compound, the decomposition products and ROS generated from redox cycling of the parent or breakdown products. We concur that the effects seen in our experiments using HaCaT cells may be mediated by absorbed quercetin, quercetin decomposition products, or both. Since the level of uptake of quercetin or decomposition products into the HaCaT cells and the antioxidant properties of the quercetin decomposition products is unknown, it is conceivable that

quercetin, the decomposition products or both may be acting as antioxidants. Nevertheless, the results of our pilot study suggest that quercetin concentrations in the 100 to 200 μM range can reduce UVR induced MMP-1 production in HaCaT cells, and that quercetin could be acting through a number of mechanisms including direct photo-absorption, intercellular antioxidant activities and alteration of cell signalling pathways.

In contrast to the HaCaT cells, the EpiDerm™ artificial skin mimics were treated topically with quercetin immediately prior to UVA and UVB exposure. Due to the immediate exposure to UVR, the quercetin would not have been absorbed by the skin and as a result would only be available to act as a physical screen or as a surface / intracellular antioxidant. In addition, the instability of quercetin observed in the HaCaT cell system was not seen as little or no loss of quercetin was observed. (Figure 7.6)

The two different routes of quercetin administration used in this study and the differences in MMP-1 and TNF-α secretion seen as a result may give some insight into the mode of action of quercetin in each case.

7.5.1 Effect of Quercetin on UVR Mediated MMP-1 Production

The quercetin-mediated concentration-dependent decrease in MMP-1 secretion seen in the EpiDerm™ treated immediately prior to exposure could result from either inhibition of MMP-1 secretion via antioxidant activity or direct photo-absorption. MMP-1 induction by UVA and UVB radiation has been shown to be caused by the formation of ROS at the cell surface[4,20-22]. As a result, both the absorbed and surface quercetin would be able to prevent initiation of MMP-1 secretion by preventing the formation of ROS at the surface of the cell, as was seen in this study. Our previous studies on UVR induced decomposition of quercetin [50] as well as other oxidation studies [53] suggest

that $^1O_2$ may interact with quercetin and this scavenging of $^1O_2$ may contribute to the quercetin-mediated reduction in MMP-1 production [23].

Quercetin may also act as a direct UVR screen, absorbing UVR and dissipating energy before ROS can be generated (note: quercetin was used at concentrations below those known to generate ROS in the presence of UVR). This absorptive effect should be localized to the surface of the skin in the EpiDerm™ samples. However, the absorbance spectrum of quercetin has a λ_{max} in the UVA region (365 nm, $\varepsilon = 28{,}400\ M^{-1}cm^{-1}$) and a low extinction coefficient in the UVB region, so it seems unlikely that the photoprotective effect would be equally strong against both UVB and UVA radiation, as is seen in this study. Our data for both the EpiDermTM and HaCaT systems suggest it is likely that the ability of quercetin to prevent MMP-1 secretion is largely an antioxidant effect in both systems.

7.5.2 Effect of Quercetin on UVR Mediated TNF-α Production

Surprisingly, we observed a dose-dependent decrease in TNF-α secretion in the EpiDerm™ study, a result that was not predicted by the HaCaT pilot study as no significant decrease was seen in the HaCaT cells. There are several possibilities to explain this observation, if the quercetin decomposition products predominate in the HaCaT cells they may have no effect on TNF-α secretion or the mechanism by which quercetin prevents secretion of TNF-α in the EpiDerm™ samples is not present in the HaCaT cells. Since there is little time for absorption to occur following the topical treatment in the EpiDerm™ samples, it is unlikely that quercetin is available to interfere with the signalling pathways activated following UVR exposure [28] leading to TNF-α

secretion. However, this leaves the possibilities of antioxidant activity and photo-absorption.

We anticipate that quercetin remains largely on or near the surface of the EpiDerm™ samples so that the antioxidant potential of quercetin is available mainly to the surface cells. Conversely we anticipate that quercetin and/or the decomposition products, and therefore their antioxidant properties, are more widely distributed in the HaCaT cell experiments. This lowered availability for antioxidant activity should result in a decreased level of protection in the skin mimics compared to the HaCaT cells. However, we did not observe this, rather a decrease in TNF-α secretion was seen only in the EpiDerm™ skin mimics.

TNF-α is known to be formed as a result of the formation of thymine dimers by a UVR-induced photo-reaction [34,35]. This is a direct photo-reaction caused by absorption of light by DNA, primarily thymine (λ_{max} = 264 nm), and does not go through a ROS intermediate. In fact, DNA damage caused by reaction with ROS results in the formation of DNA lesions distinct from thymine dimers which have not been shown to cause TNF-α production [34,35]. This causative relationship between thymine dimer formation and TNF-α production further supports the conclusion that in the case of the decreased TNF-α secretion seen in the EpiDerm™ samples, direct UVR absorption is the major protective mechanism of quercetin. A strong absorbance band of quercetin (λ_{max} = 256 nm, ε = 28,300 $M^{-1}cm^{-1}$) is near that of thymine, while the major decomposition products previously identified for quercetin show little absorbance in the UVC region, instead having absorbance maxima near 300 nm [50,53]. Thus, if the decomposition products predominate in the HaCaT cells, they would be anticipated to provide a weaker

absorptive effect than quercetin. In addition, the topical treatment immediately prior UVA and UVB exposure should provide a greater level of protection than the pre-exposure of the HaCaT cells as the cell media containing quercetin is removed from the cell cultures prior to UVR exposure. This leaves only that quercetin which has been absorbed by the cells and remaining near the cell surface available to offer photoprotection by light absorption. A decrease in the effectiveness of the photo-protection offered by quercetin would be expected in the pre-treated HaCaT cells as opposed to the topically treated EpiDerm™ cells which is in agreement with our results.

Using the artificial skin mimic EpiDerm™, we have demonstrated that topically applied quercetin decreases two markers of UVR-induced skin damage in a concentration-dependent manner. These markers were selected to be indicators of UVR-induced ROS (MMP-1) and direct UVR absorption by DNA (TNF-α). Use of HaCaT cells as a pilot study for the EpiDerm™ experiments resulted in a quercetin-mediated decrease of MMP-1, but no effect on TNF-α, which we attribute to the instability of quercetin in cell media such as DMEM. Our results are in agreement with quercetin providing a photoprotective effect both as an antioxidant and through absorption of UVR. Previous studies on the apoptotic properties of quercetin in UVR treated HaCaT cells suggested that stabilizing quercetin was not necessarily beneficial [52]. While we observed different markers of biological damage than Bowden's group, our results agree with studies on topical quercetin formulations which suggest the importance of quercetin stability in the prevention of UVR-induced skin damage[54].

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8. Summary and Conclusions

The overall aim of this research project was to assess the utility of the natural product quercetin as a topically applied sunscreen and in order to accomplish this goal four specific targets were set. First, the ability of quercetin to prevent lipid peroxidation in a model lipid membrane system was quantified using both a chemical oxidant as well as UVA and UVB radiation to initiate oxidation. Second, the photostability of quercetin was determined and the photodecomposition products were identified. Third, the ability of quercetin to prevent pharmaceutical photosensitization by the drug ketoprofen was assessed by determining if quercetin could prevent photodecomposition of ketoprofen and if so what happened to quercetin during the process. Fourth, the ability of quercetin to prevent the generation of MMP-1 and TNF-α, markers of photoaging and UVR induced DNA damage respectively was assessed in both HaCaT cell cultures and EpiDerm artificial skin mimics.

The following sections summarize the findings of this study and put these findings into the context of the project as a whole.

8.1 - Determination of the anti-oxidant capacity of quercetin in a simulated membrane system

The measurement of quercetin's antioxidant stoichiometry in DLPC lipid membranes showed that each molecule of quercetin is capable of scavenging four reactive radical species, making quercetin a highly effective antioxidant. Since many of the negative effects of sunlight on skin are caused by the generation of ROS, this indicates that quercetin may be effective in preventing photo-oxidative skin damage. Interestingly, the stoichiometry of quercetin was the same for oxidation initiated by UVB and for oxidation initiated by the chemical oxidant AAPH. This indicates that quercetin's ability to protect

the model membranes against UVB induced oxidation is due to its ability to scavenge radical species and not due to any direct absorption of UVB radiation. Due to quercetin's relatively weak absorbance in the UVB range, this result is not surprising, especially given quercetin's strong anti-oxidant ability. More surprising is that when lipid peroxidation was initiated by UVA radiation quercetin offered less protection, showing an anti-oxidant stoichiometry of only 2. This result was unexpected since quercetin has an absorbance maximum in the UVA range at a wavelength of 365nm. We speculate that the increased absorption of energy in the UVA range results in quercetin forming a high energy intermediate with decreased anti-oxidant capacity and possible pro-oxidant activity. In spite of the decreased effectiveness of quercetin as an antioxidant against UVA, it still offers some degree of protection, scavenging two reactive radicals for each molecule of quercetin. Taken together the data from the first set of experiments indicates that quercetin is an effective protectant of lipid membranes against UVB, and to a lesser extent UVA, induced peroxidation by acting as a potent radical scavenger. Since many of the negative skin effects associated UVR exposure of skin are mediated through reactive species, the strong anti-oxidative activity of quercetin indicates good potential for use as a topical photoprotectant.

8.2 - Determination of the photostability of quercetin and identification of quercetin's photodecomposition products

The photostability of quercetin was determined in both methanol and a methanol-water mixture and in the presence of both UVA and UVB radiation. Quercetin was found to have good photostability with less than 20% loss of quercetin following 11 hours of exposure. Since exposure to UVR at the intensities used in the experiment for a total of 11 hours is highly unlikely if not impossible in a normal usage situation, the loss of

quercetin seen in our studies is not a large negative influence on its utility as a photoprotectant. In fact, the rate of decomposition of quercetin was so slow that a photosensitizer, benzophenone, had to be added in order to generate sufficient product for identification. However, the formation of decomposition products, even in small amounts, requires that the products be identified. HPLC-PDA and HPLC-MS analysis demonstrated that the same three photoproducts were formed when either UVA or UVB radiation was used and that the presence of the photosensitizer benzophenone accelerated the decomposition of quercetin but did not change the three products formed. In addition, the experiment was carried out under reduced oxygen conditions to determine what role if any oxidative reactions via ROS played in the decomposition process. Under reduced oxygen the same three products were formed though in a different ratio then under atmosphere.

The three photoproducts of quercetin were identified to be 2,4,6-trihydroxybenzaldehyde, 2-(3',4'-dihyroxybenzoyloxy)- 4,6-dihydroxybenzoic acid (quercetin depside) and 3,4-dihydroxyphenylethanol (hydroxytyrosol). These three products remained consistent when either UVA or UVB radiation was used and when quercetin was dissolved in methanol or a methanol-water mix. Although the three products remained the same regardless of the presence or absence of sensitizer or levels of oxygen available, the ratios of the three products changed. In the case of UVR exposure in the presence of oxygen without a sensitizer, photoproduct 2 – quercetin depside, an oxidation product of quercetin – was favoured. However, in the presence of the triplet state sensitizer benzophenone or under reduced oxygen conditions the extent of formation of product 3 was equal or greater than the formation of product 2. This led us

to hypothesize that two separate product formation pathways are present. In the presence of oxygen a type 1 photoreaction is favoured where ROS, likely singlet oxygen, is formed and reacts with quercetin to form the oxidation product quercetin depside. At the same time direct absorption of radiation by quercetin results in decomposition of quercetin resulting in products 1 and 3, each of which is one half of the parent molecule. The photosensitizer benzophenone mimics the direct absorption reaction in that the excitation energy is passed directly to the quercetin molecule resulting in cleavage of quercetin.

A literature search of these three decomposition products showed that there was minimal toxicity associated with any of them and that formation of them on the skin was unlikely to cause any detrimental health effects. The favourable stability of quercetin exposed to light along with the non-toxic nature of what decomposition products are formed indicates that quercetin is a good candidate for a topically applied sunscreen.

One interesting aspect of the photoproducts of quercetin is found in their UVR absorbance spectra. Quercetin itself has two maximal absorbance bands in the UVA and UVC ranges with a minimum in the UVB range. This results in quercetin offering less protection by direct absorption in the high energy UVB while maximum protection occurs in the less energetic UVA range (note that very high energy / highly damaging UVC is filtered by the ozone layer in the atmosphere). However, the photodecomposition products of quercetin all have absorbance maxima in the UVB range due to the loss of conjugation across the whole molecule. This led us to speculate that in plants where quercetin has been shown to be photoprotective, it may be that quercetin alone is only somewhat protective (against UVA) and that the plants rely on the formation of the photodecomposition products to provide protection from the UVB. This

hypothesis is outside the scope of the current project as well as the expertise of the researchers currently involved with the project, but we believe it is an interesting possibility. In addition, this speculation provides ideas for other compounds to be tested as sunscreens in the future.

8.3 - Determination of quercetin's ability to prevent photosensitization due to the decomposition of the xenobiotic ketoprofen.

During the course of the project, it came to our attention that quercetin had been shown to prevent breakdown of the topical NSAID ketoprofen (UVR-induced ketoprofen decomposition is known to lead to photosensitization), although the mechanism by which this protection occurs was not elucidated. Due to the structural similarities between ketoprofen and the photosensitizer we had been using, benzophenone, we speculated that quercetin may be preventing ketoprofen photosensitization through transfer of the excitation energy from ketoprofen to quercetin, resulting in the formation of the quercetin photodecomposition products we had identified. This was found to be the case, as we found that ketoprofen was spared from photodecomposition as a long as quercetin was present. However, this sparing of ketoprofen resulted in the loss of quercetin through the formation of the photoproducts we had earlier identified, and once the quercetin was consumed ketoprofen degraded as observed by other researchers, possibly leading to photosensitization.

The possibility that quercetin could have a protective effect not only against direct effects of UVR on the skin but also through the prevention of xenobiotic induced photosensitization raises the possibility of using quercetin in conjunction with pharmaceuticals known to induce photosensitization, hopefully preventing these adverse reactions. However, before any more conclusions can be made in this area the ability of

quercetin to prevent the decomposition of other products needs to be tested, a set of experiments which falls outside the scope of this project.

8.4 – Determination of the effect of quercetin on biomarkers of photoaging and photoinduced DNA damage.

Two of the major negative effects of UVR on human skin are the induction of photoaging and damage to DNA, which has the potential to lead to skin cancers. In order to assess the effect of quercetin on these endpoints we chose two biomarkers, MMP-1 for photoaging and TNF-α for DNA damage, and assessed the effects of quercetin in HaCaT cell cultures and EpiDerm artificial skin mimics. It was found that quercetin applied to either system at concentrations as low as 100μM resulted in a decrease in secretion of MMP-1, indicating that quercetin should be an effective protectant against UVR induced photoaging. The effects of quercetin on TNF-α were mixed, with no effect being seen in the HaCaT cell, but a significant reduction in TNF-α in the EpiDerm samples indicating a decrease in DNA damage, specifically thymine dimer formation. The differences in the TNF-α response between the two sample types may be explained by the difference in the routes of administration since in the HaCaT cells the quercetin was administered 24 hours before UVR exposure in the media while for the EpiDerm the quercetin was applied topically immediately before exposure. Quercetin has been shown to be unstable in cell media which may mean that very little was left to offer protective effects at the time of UVR exposure of the HaCaT cells resulting in no protection for DNA damage while the topically applied quercetin was present to prevent DNA damage in the EpiDerm samples. The goal of this project was to assess the use of quercetin as a topical photoprotectant, which means that EpiDerm experiments are more relevant to the mode of application we are proposing. Since quercetin showed significant protective effects against both

photoaging and DNA damage in the EpiDerm models, we have concluded that topically applied quercetin is an effective photoprotectant against these negative effects of UVR.

Use of topically applied quercetin in the EpiDerm samples also addressed another possible problem with the use of quercetin as a sunscreen. Quercetin has a distinct bright yellow colour and the concern existed that topical application of some formulation containing quercetin at an effective concentration would impart an undesirable yellow colour to the skin. However, the EpiDerm experiments showed that use of quercetin at concentrations that provided protection against UVR-induced biomarkers resulted in no noticeable yellow colouration being imparted to the skin mimics meaning no negative cosmetic effect is present.

It is interesting to note that while the HaCaT experiments were initially preformed to establish the effectiveness of the end-points chosen and determine a general range of concentrations to be tested in the EpiDerm samples, the differences in the mode of application of quercetin may provide some insight into quercetin's mechanism of action. There are three main mechanisms by which quercetin may act as a photoprotectant, direct absorption of UVR, scavenging of reactive species such as ROS, alteration of metabolic pathways or a combination of the three. Since the HaCaT cells were dosed 24 hours prior to UVR exposure with quercetin in the media and the media was removed prior to UVR exposure, only quercetin or its decomposition products which had been absorbed into the cell could exert an effect. As a result the most likely mechanisms of action seen in these samples are through antioxidant effects or metabolic pathway effects since the amount of light absorbed by quercetin within the cell would be minimal. In contrast, the quercetin applied topically to the EpiDerm samples immediately prior to UVR exposure would be

most likely to act through direct absorption of UVR or cell surface antioxidant activity since the short exposure time would make absorption and interaction with cellular pathways unlikely.

Since a significant decrease in MMP-1 secretion is seen in both HaCaT cells and EpiDerm skin mimics, it may suggest that the mechanism of action active in the prevention of MMP-1 secretion is that mechanism that is shared by both sample types, namely antioxidant activity. Since production of MMP-1 is known to be the result of a cascade initiated by oxidation of cell surface proteins, this result makes sense, although the possibility that effects on cell signaling may also have an effect cannot be ignored. In contrast to MMP-1, a significant decrease in TNF-α secretion was seen only in the EpiDerm samples, not the HaCaT cells. This result suggests that the mechanism of action here is that which is present in the EpiDerm samples but lacking in the HaCaT cells, namely direct absorption of UVR. Again this makes some sense as TNF-α production is known to be the result of thymine dimer formation which is a direct UVR induced form of DNA damage not acting through ROS. As such, thymine dimer formation, and therefore TNF-α secretion, should not be affected by antioxidant activity only by an actual decrease in the amount of UVR reaching the cell. However, these conclusions are speculative at best and full elucidation of the mechanisms involved would require a number of experiments beyond the scope of this project.

8.5 - Conclusions

The primary goal of this research project was to assess the effectiveness of topically applied quercetin as a possible sunscreen. We have shown that quercetin has a number of characteristics that make it a good candidate for topical sunscreen use. First, quercetin

shows good stability to both UVA and UVB radiation with less than 20% loss of parent over a period exceeding normal sun exposure. In addition the products formed by decomposition of quercetin are not known to be toxic. Second, quercetin has been shown to be an effective antioxidant scavenging four radical species for each molecule of quercetin when oxidation is initiated by UVB. Although this number drops to two radicals in the case of UVA, this still makes quercetin an effective antioxidant. Third, quercetin has been shown to prevent the decomposition of ketoprofen which can lead to photosensitization through the deactivation of ketoprofen, resulting in the formation of the previously identified non-toxic decomposition products raising the possibility that quercetin could be used in a formulation for topically applied pharmaceuticals that are either phototoxic or show photodegradation. Fourth, when quercetin was applied topically to skin mimics there was a significant decrease in the secretion of MMP-1 and TNF-α, markers of photoaging and photoinduced DNA damage respectively, two of the major deleterious effects of sunlight on skin. In total, these finding provide evidence that topically applied quercetin is an effective form of photoprotection.

In addition to the primary goal of assessing the photoprotective potential of quercetin, this project also had a secondary goal of developing a series of analytical techniques with which other possible photoprotectants could be evaluated. The techniques that have been used in this project have now been well established and are available for assessment of other compounds.

9. Future Work

Although the primary and secondary research goals of this project have largely been achieved, and number of additional questions remain to be answered.

9.1 Formulation

Quercetin has been shown to be an effective photoprotectant, but the method of application used in this study, application of quercetin dissolved in acetone, is not suitable for commercial use due to the drying effects of acetone. If use of quercetin as a sunscreen or a component of a sunscreen formulation is to be achieved a useable matrix for application such as an inert cream would need to be found and evaluated.

9.2 Non-surrogate DNA endpoint

In the EpiDerm and HaCaT experiments, MMP-1 and TNF-α were used as biomarkers for photoaging and DNA damage respectively. MMP-1 secretion is an effective end-point, but TNF-α could be replaced with a more direct endpoint. An HPLC-MS-MS method for the determination of thymine dimers in cells was partially developed but validation for analysis in cell extracts was not achieved. Complete development and validation of this method would be an asset in evaluation of future sunscreen candidates.

9.3 Prevention of photosensitization by other xenobiotics

Quercetin was shown to prevent the photodecomposition of ketoprofen, a reaction causing photosensitization, through absorption of excitation energy. Assessment of the ability of quercetin to prevent photosensitization by other drugs or environmental contaminants could demonstrate even greater utility for quercetin as a sunscreen. The prevention of photosensitization would have to be measured in terms of quercetin's

ability to both prevent photodecomposition or excitation of the sensitizer and in terms of prevention of negative biological end-points.

9.4 Mechanism of action

Although some possible mechanisms of action for the photoprotective action of quercetin have been suggested here, these are on suppositions. Clear elucidation of the mechanisms involved would require a large number of experiments in which the other mechanisms were blocked in order to confirm the suspected mechanism is the active one. However, such experiments would provide valuable information on the effects of quercetin and related compounds on the skin and on the body in general.